"STUDIES ON OXYTOCINASE ACTIVITY IN HUMAN PREGNANCY"

by

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"You must always let yourself think about everything. And you must think about everything as it is, not as it is talked about."

G.B. Shaw

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INTRODUCTION

EXPERIMENTAL PART

I N T R O D U C T I O N

HORMONES OF THE POSTERIOR LOBE OF THE PITUITARY GLAND

1 - HISTORY

Our knowledge of the endocrine functions of the pituitary gland is comparatively recent. Text-books of physiology published in the late 19th century (e.g. Michael Fost Text-Book of Physiology, 1891 edition) gave a good histological description of the gland, emphasised its ductless nature, but could attribute no definite function to it.

The first evidence suggesting any function for the pituitary body was presented by Oliver and Schäfer in 1895. They observed that extracts from the whole pituitary gland had a pressor activity when injected intravenously into the anaesthetised dog.

Howell (1898) showed that the substance responsible for this pressor action of pituitary extracts was obtainable only from one part of the organ, and rather surprisingly, only from its posterior, neural lobe. At the time this appeared to be quite an anomalous finding, as the main glandular structures were known to be localised to the anterior lobe of the pituitary.

Dale (1906) discovered the oxytocic action of posterior pituitary extracts by mere chance. Whilst investigating the sympatholytic action of certain ergot alkaloids he recorded the uterine activity as well as the arterial blood pressure of a pregnant female cat.

The effects of adrenaline on both target organs were reversed by a previous dose of the ergot preparation. For the sake of comparison, Dale then injected a dose of a posterior pituitary extract, when he noticed that it had not only retained its normal pressor action but it also produced a profound contractile response of the uterus.

The milk-ejecting properties of the posterior pituitary extracts were demonstrated a few years later by Ott and Scott (1910). However, the greatest controversy centred around the effects on the rate of urine secretion. Magnus and Schafer (1901) observed that an intravenous injection of the pituitary posterior lobe extracts into an anaesthetised animal produced, after a latent period, a conspicuous increase in the rate of flow of urine; i.e. a diuretic response. This observation was confirmed by Schafer and Hering (1906) and Dale (1909). Later, Schafer (1909) subjected the pituitary glands of dogs to thermo-cautery, and noted that polyuria supervened during the post-operative period. Frank (1912) discovered a strong pathological association between human diabetes insipidus and disease or injury of the neurohypophysis. He attributed the disease to excessive pouring of the supposed diuretic hormone evoked by irritation of the neurohypophysis. Farmi (1913) and Van den Velden (1913) independently investigated the effect of injection of posterior

pituitary extracts on the course of the disease in cases of diabetes insipidus. They both reported a favourable result which cast doubt on the earlier diuresis finding of Schafer and Frank's application of this idea to his pathological study. Konschegg and Schusten (1915) re-investigated the problem in conscious animals and proved that the true action of posterior pituitary gland extracts on the kidney is antidiuretic. The earlier finding of Schafer, was recognised to be an artefact resulting from the depression of kidney function produced by anaesthesia, and to the injection of comparatively large doses of the extract by the intravenous route.

The earlier concept by Dale (1909) that these different effects of pituitary extracts were due to one and the same hormone was also subsequently disproved by fractionation of the extracts into two constituents, of which one is mainly oxytocic and the other is vasopressor and antidiuretic (Dudley, 1919). Separation was achieved on a commercial scale by Kamm et al (1928) in the laboratories of Park Davis and Company. Since then, preparations have been available for clinical use of comparatively pure oxytocin (pitocin) and vasopressin (pitressin).

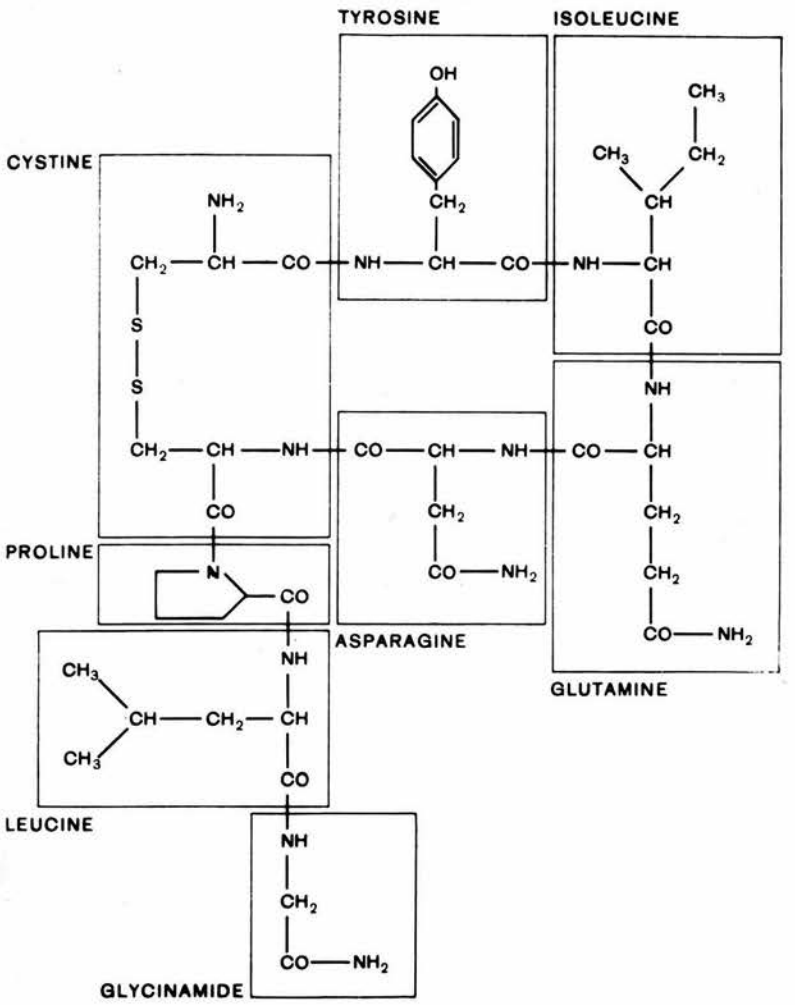
In 1949, Livermore and Du Vigneaud were able to isolate the hormones in a chemically pure form from the pituitary extracts of animals. This was the starting point for elucidating their molecular structure. The

chemical formula of oxytocin was worked out independently by Du Vigneaud et al (1953)$_a$ in New York, and Tuppy (1953) in Vienna, employing completely different degradation techniques. Tuppy published his finding a few weeks before Du Vigneaud et al. However, Du Vigneaud and his co-workers (1953)$_b$ were also able to prove the formula by synthesising the hormone. This was the first polypeptide hormone to be synthesised, and for this Du Vigneaud was awarded the Nobel prize for chemistry in 1955. The chemical formula for vasopressin was subsequently established by Du Vigneaud et al (1953)$_c$ and the proposed structure was also proved by synthesis.

It must be emphasised that advance in oxytocin and vasopressin research was comparatively rapid when compared to other polypeptide hormones e.g. insulin. This is largely due to the fact that the former hormones are easily extracted from mammalian posterior pituitaries by dilute acetic acid in which they are very stable. Such simple extracts were employed by Bell in obstetric practice as early as 1909.

2 - CHEMISTRY AND SYNTHETIC ANALOGUES

Chemically, human oxytocin and vasopressin proved to be relatively simple octapeptides (molecular weight approximately 1000) with closely interrelated structures (Figs. 1 and 2). The approved numbering of the individual



The structural formula of oxytocin.

Figure 1

amino acid residues is the one suggested by Bodansky and Du Vigneaud (1959) and is shown in Fig. 2. Thus, human oxytocin and vasopressin share six common amino acid residues with differences only in position 3 (isoleucine in the oxytocin molecule being replaced by phenylalanine in vasopressin), and position 8 (where an arginine residue in vasopressin replaces leucine in oxytocin).

Investigation of neurohypophysial hormones in posterior pituitary extracts of various animal species has so far revealed the presence of some other peptide variants, namely:

a Lysine - Vasopressin in the hog (Fig. 2)

b Vasotocin (3-I-leucine, 8-arginine oxytocin in birds, frogs and fishes (Fig. 2)

c Isotocin (3-I-leu., 4-serine, 8-I-leucine oxytocin) in fishes (Fig. 2)

All natural posterior pituitary hormones thus seem to share five common amino acids, showing differences only in positions 3, 4 and 8 of the molecule. They also share the following chemical features:-

1 A disulphide bond between the two half-cystine residues in positions 1 and 6. Disruption of this bond by reduction or oxidation leads to loss of biological activity.

2 A cyclic pentapeptide ring involving the residues 1 to 6, and comprising exactly 20 carbon atoms.

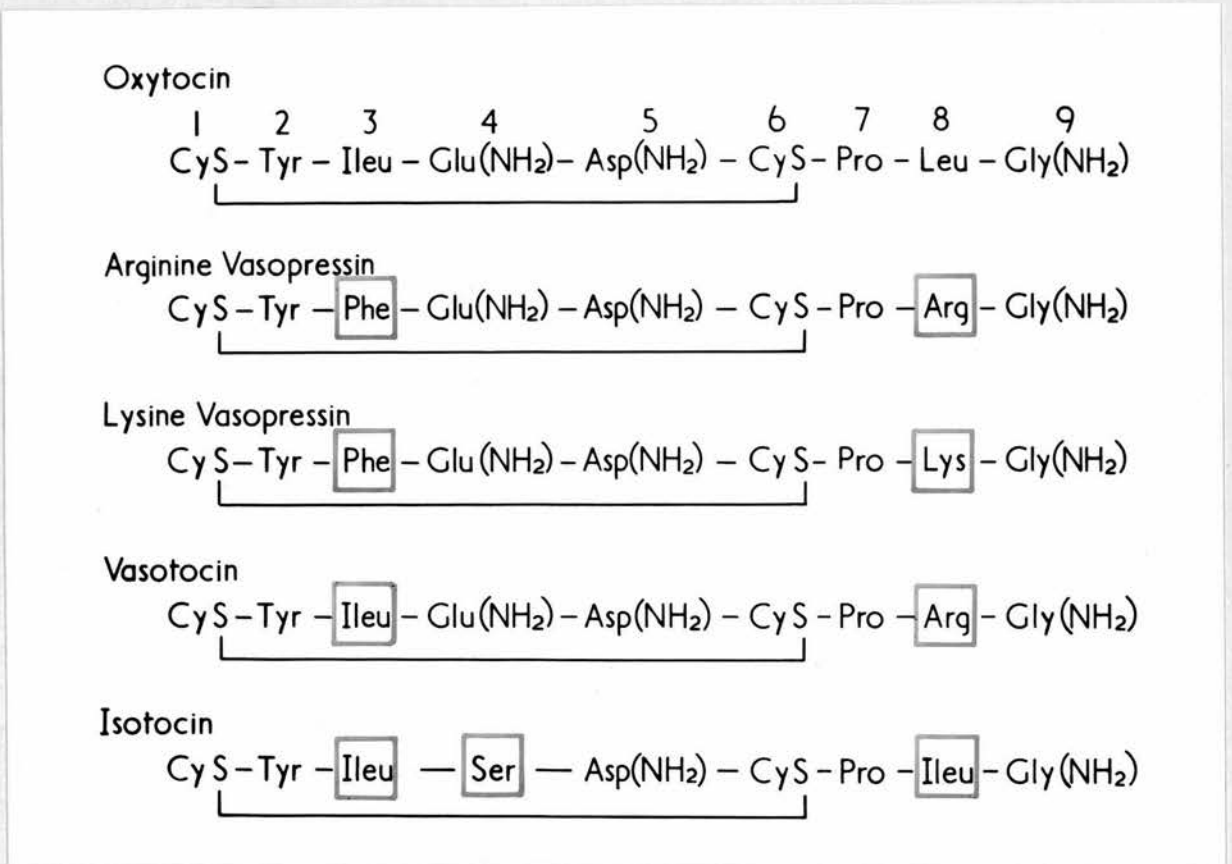


Figure 2 Amino Acid Sequence of Polypeptides from the Posterior Pituitary Glands of Man and Animals.

3 A free amino group in position 1.

Although the differences in the chemical structure of the natural hormones are relatively small, the above peptides show vast contrasts in biological activities as evaluated by various pharmacological techniques. This fact has stimulated research in the field of synthetic analogues of oxytocin and vasopressin. Over the past decade more than a hundred of these analogues have been synthesised and assessed biologically, mainly by Du Vigneaud and co-workers, Biossonnas et al (1956), and by Rudinger (1964). As pointed out by Berde (1964), the aim of these studies is

a To study the relation between chemical structure and biological activity. e.g. it is now established that a basic amino acid in position 8, such as arginine, lysine or histidine, enhances the anti-diuretic effect of the molecule.

b To produce polypeptides with modified spectrum of activity which are useful for specific clinical purposes and are free from undesirable side effects.

c To produce compounds which prove to have antagonistic properties to oxytocin and vaso-pressin for clinical trial.

So far the only synthetic analogues which have proved to be of clinical value are Octapressin (Berde, 1964) and 1-Desamino-oxytocin (Du Vigneaud et al, 1960)

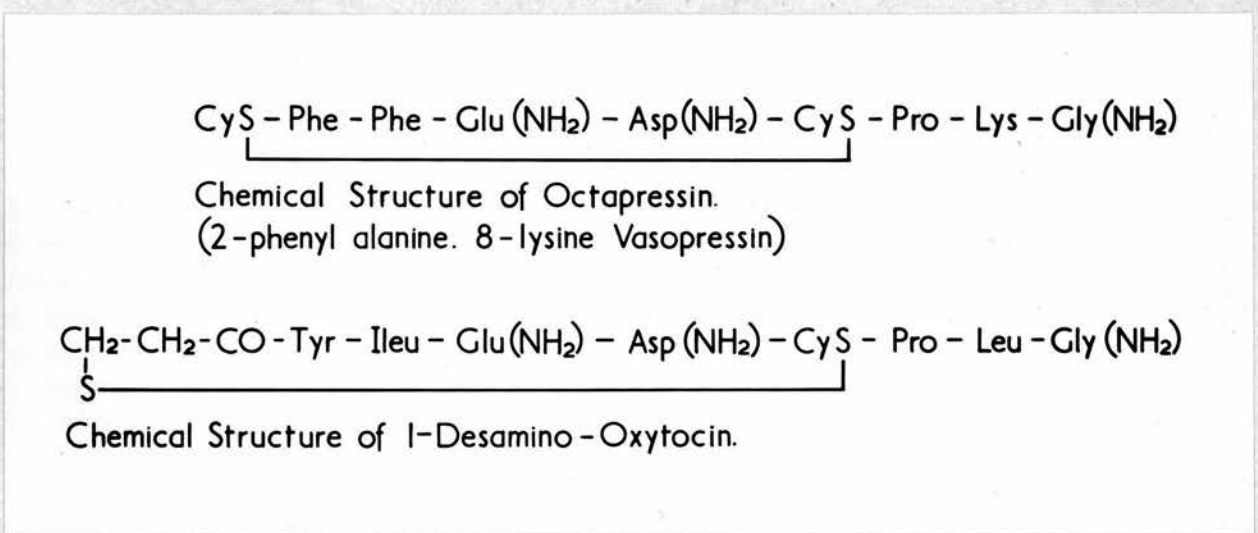


Figure 3 **Synthetic Analogues of Oxytocin of Therapeutic Value.**

which are shown in Fig. 3. Octapressin has a marked vasoconstrictor effect with little antidiuretic or oxytocic action and is useful as a haemostatic agent in surgical and gynaecological operations. 1-Desamino-oxytocin has a high oxytocic activity on the human uterus (Embrey, 1965), and per unit weight it is almost twice as potent as oxytocin itself.

3 - METHODS OF ASSAY

In spite of the great advances in the chemistry of neurohypophysial hormones, there is yet no chemical method for their estimation. Various pharmacological preparations are thus employed for their biological assay. Tables 1 and 2 summarise the biological assay methods for oxytocin and vasopressin respectively.

In all tests unknown quantities of the hormones are matched to doses of an International Standard (or a substandard derived from it), and the result is expressed in international units. By definition one international unit of either oxytocin or vasopressin is equivalent to the activity of 0.5 mg. of a standard acetone dried preparation of ox posterior pituitary gland. This corresponds to approximately 2 μg. of the pure synthetic hormones.

The assay of oxytocin and vasopressin in blood and other biological fluids presents many technical difficulties (Walker, 1960). These include

a The physiological concentration of these hormones

TABLE 1

BIO-ASSAY METHODS FOR OXYTOCIN

Test Animal	Method	Approximate sensitivity in m.U.	References
Rat	Contraction of uterus in vitro		
	a - Isolated uterus	2-3	Holton, 1948
	b - Superfused uterus	0.002-0.008	Fitzpatrick, 1961
Fowl	Lowering of blood pressure	100	Coon, 1939
Lactating Rabbit	Milk ejector effect		
	a - Intravenous injection	2-3	Van Dyke et al, 1955
	b - Injection into mammary artery	0.08-0.30	Fitzpatrick, 1961
	c - In vitro contraction of mammary strip	0.150	Mendez-Bauer, et al, 1960
Guinea Pig	Milk ejector effect		
	a - Injection into mammary artery	0.005-0.010	Tindal and Yokoyama, 1962
	b - Intravenous injection	0.05-0.30	Tindal and Yokoyama, 1962

TABLE 2

BIO-ASSAY METHODS FOR VASOPRESSIN

a Antidiuretic Activity

Test Animal	Method	Approximate sensitivity per animal m.U.	References
Dog	Unanaesthetised, water loaded	0.025-0.05	Theobald, 1934
	Hypophysectomised, unanaesthetised, water loaded	0.2-0.3	Hare et al, 1945
Rabbit	Paraldehyde anaesthesia, water loaded	0.5	Walker, 1939
Rat	Unanaesthetised, water loaded	1-5	Burn, 1931; Ginsburg, 1951
	Ethanol anaesthesia, continuous water load, doses injected I/V	0.007-0.100	Dicker, 1953
Mouse	Water load. No anaesthesia	0.050	Heller and Blackmore, 1952

b Pressor Activity

Test Animal	Method	Approximate sensitivity per animal m.U.	References
Dog	Chloretone anaesthesia	-	Hamilton and Rowe, 1916
Cat	Spinal preparation, ether anaesthesia, brain destruction, artificial respiration	-	Hogben and Schlapp, 1924
Rat	Barbiturate anaesthesia, pithing of spinal cord	4-20	Landgrebe et al, 1946; Debaniski, 1951
	Urethane anaesthesia, hypotensive agent	-	British Pharmacopoeia, 1963

in peripheral venous blood is very low and is beyond the sensitivity of the usual assay methods. This difficulty can be overcome by:

1 The use of hypersensitive methods for assay, e.g. superfused rat's uterus for oxytocin estimation.

2 Collection of central venous blood from the jugular veins into which the pituitary secretions drain and would thus be present in higher concentrations. In rat and sheep the external jugular vein drains most of the blood from the neurohypophysis and is relatively easy to approach. In other species including man, internal jugular venous blood is required.

3 Extraction and concentration of the hormones from the biological media before the final assay. Several methods have been described, including acid ethanol extraction (Bisset, 1961) for whole blood; acetone extraction for serum (Fitzpatrick, 1961); and gel filteration of plasma on saphadex G-25 columns (Folley and Knaggs, 1965). These methods involve a variable loss of activity. In addition, the first two commonly lead to concentration of interfering substances which affect the assay preparation.

b Biological fluids commonly contain interfering substances which affect the biological preparations used for assay. Thus the isolated rat uterus preparation

commonly used for assay of oxytocin is affected by excess of potassium ions, adrenaline, serotonin, and vasopressin, all of which are present in blood. Plasma kinins, which similarly affect the rat uterus, are released on contact with glass containers used for collection. To minimise fallacious readings from these non-specific interfering substances, the following precautions are commonly used:

(i) Collection of samples in plastic pre-cooled containers.

(ii) Dialysis of material before assay.

(iii) Use of thioglycollate to ascertain that the noticed activity is due to oxytocin or vasopressin. Thioglycollate in appropriate molar concentration disrupts the disulphide bond of the active peptides and leads to loss of their biological activity. Biological activity not abolished by thioglycollate is considered not due to oxytocin or vasopressin.

(iv) Chromatography of the final extract on paper with a control run of the pure active principle. The active polypeptide in the extract is identified either by staining, or by elution of the area corresponding to the control followed by biological assay of the eluate.

Recently Gilillard and Thaddeus (1965) explored the antigenic properties of oxytocin in the rabbit, and could demonstrate specific antibody formation. Their work indicates the feasibility of an immuno-assay approach to posterior pituitary hormones.

4 ORIGIN, STORAGE AND RELEASE

There is ample histochemical and electronmicroscopic evidence that oxytocin and vasopressin are actually produced in the hypothalamic nuclei, carried along the neurone axons in the pituitary stalk as stainable neurosecretory material, and only stored in relation to the nerve endings (pituicytes) in the posterior lobe of the pituitary gland (Scharrer and Scharrer, 1954). There is no agreement, however, on their exact form of existence in the neurohypophysis. Van Dyke et al (1941) isolated a protein substance (molecular weight 30.000) from beef posterior pituitary glands which contains oxytocin and vasopressin in a fixed 1:1 ratio. The active protein cannot be removed from Van Dyke protein by dialysis against water, but can be liberated by dialysis against 0.1 N acetic acid, electrodiolysis, or trichloracetic acid precipitation (Acher and Fromageot, 1959). Since these gentle processes do not hydrolyse peptide bonds, it appears unlikely that the active peptides are fragments of the Van Dyke protein. It is more likely that the protein represents the "carrier substance"

to which the peptides are bound in a relatively loose association during transport and storage within the neurohypophysis.

Release of the active hormones from the gland can be affected by a wide variety of stimuli including:

1 Direct electrical stimulation of the posterior lobe of the pituitary gland and the associated neurohypophysial-hypothalamic tracts.

2 Administration of drugs, notably nicotine, acetylcholine, hexamethonium, morphine, many tranquillisers and anaesthetic agents. Ethyl alcohol, on the other hand, inhibits release of the endogenous hormones and is widely employed for anaesthetising animals for their in vivo biological assay. Hypertonic intravenous infusions of saline are also a potent method for stimulating release of the hormones of the posterior pituitary, probably through stimulation of osmoreceptors in the hypothalamic region.

3 Physiological stimuli include dehydration (probably by increase in plasma osmolarity), suckling, probably during childbirth, and possibly during mating. Reduction of the circulatory volume by haemorrhage or shock can also be an effective stimulus.

There is no agreement on the selective release of the individual hormones. Effective stimuli in most instances appear to provoke the release of both oxytocin

and vasopressin into the circulation, commonly in a fixed ratio with oxytocin invariably in excess to vasopressin (Pickford, 1960). However, there is some evidence on the separate release of the two hormones individually in human experiments (Theobald, 1959; Cobo et al, 1965).

There is also some doubt about the form in which the hormones are carried in the blood. Thorn and Silver (1957) suggest that neurohypophysial peptides are partially bound to plasma proteins in an easily dissociable form. Ginsburg and Smith (1959) observed however, that injected doses of peptides diffuse into volumes approaching total body water indicating that such binding is neither complete nor irreversible. A similar finding was reported by Heller (1957).

THE ROLE OF OXYTOCIN AND VASOPRESSIN IN REPRODUCTIVE PHYSIOLOGY

1 OXYTOCIN AND NATURAL CHILDBIRTH

Oxytocin is known to accelerate labour by eliciting or re-inforcing contractions of the uterine smooth muscle in many species including man. This property finds wide clinical application both for induction of labour (Theobald et al, 1948), and for treatment of uterine inertia in labour. This action of oxytocin is mediated through a direct effect on receptors in the smooth muscle cells of the uterus and can be demonstrated in vivo as well as in vitro. From electrophysiological

studies on rat uterus (Jung, 1961) and rabbit myometrium (Csapo, 1961) it appears that the primary action of oxytocin on the uterine muscle is to lower the membrane potential. In high concentrations, oxytocin also exerts a direct effect on the contractile system of the myometrial cells, even when the membrane is depolarised (Evans et al, 1958).

The sensitivity of the uterus to neurohypophysial hormones depends on many factors, including species, hormonal and reproductive status and ionic milieu (Van Dyke, 1961). There is general agreement that in human pregnancy there is progressive rise in myometrial sensitivity to oxytocin (Pose and Caldeyro-Barcia, 1958, and Fitzpatrick, 1957). According to Caldeyro-Barcia, the sensitivity reaches a maximum in the last four weeks of pregnancy, with no further change occurring in pre-labour days. Smyth (1958) on the other hand, reported a sharp increase in uterine sensitivity to oxytocin in the few days preceding labour. Although the techniques employed were different (Pose and Caldeyro-Barcia assessed the myometrial sensitivity from the uterine response to various doses of oxytocin administered in a continuous intravenous drip, whereas Smyth injected oxytocin in interrupted doses of 10 mU/minute) there is still no plausible explanation for the different results.

The increased uterine sensitivity to oxytocin in pregnancy is generally attributed to the existing high levels of oestrogens. Oestrogens are known to increase

the sensitivity of the myometrium to oxytocin (Csapo, 1961; Schofield, 1957).

The role of progestrone in suppressing uterine reactivity to oxytocin is subject to considerable controversy and apparently shows wide species differences. The rabbit represents a classical example of a mammal in which progestrone renders the myometrium refractory to oxytocin (Csapo, 1961). In human pregnancy, however, large doses of progestrone fail to decrease the sensitivity of the uterus or to delay the onset of spontaneous labour (Pose and Fielitz, 1961). There is also no evidence that progestrone levels in blood drop in late pregnancy or at the time of labour (Short, 1960) when the reactivity of the myometrium is at its zenith. Throughout pregnancy, the human uterus shows a progressive sensitivity to oxytocin in spite of rising progestrone levels. This is in sharp contrast with the rabbit uterus, which became responsive only in the last few days of pregnancy (Csapo, 1961). In 1956, Csapo evolved the theory of a local progestrone block, upholding the view that the effect of progestrone in human pregnancy can be exercised by a local inhibitory effect on the myometrium at the placental site. The block is raised at term, allowing the onset of natural childbirth.

The possible physiological role of oxytocin in natural parturition is suggested by the following evidence:

1 There is complete analogy between normal

spontaneous uterine contractions and uterine motility elicited by oxytocin infusion, appropriately administered in correct dosage. This applies to the shape, intensity, frequency, and tone as well as co-ordination of the contractions (Caldeyro-Barcia and Poseiro, 1958).

2 Occurrence of milk-ejection in lactating women (Gunther, 1948) and rabbits (Cross, 1958) with each uterine contraction during labour. Similarly, Caldeyro-Barcia (1960) recorded the intra-mammary duct pressure in women during labour, and reported increased rhythmic activity implying release of oxytocin.

3 Depletion of the oxytocin content of the neurohypophysis in various laboratory animals during labour, again suggesting a rapid drain (Dicker and Tyler, 1953; Acher and Fromageot, 1957).

4 The controversy as to whether or not labour was retarded by hypophysectomy or section of the pituitary stalk (Reviewed by Harris, 1955) has largely lost its interest, since it is now known that oxytocin and vasopressin are produced in the hypothalamic nuclei. These are now regarded as part of the neurohypophysis as the posterior lobe of the pituitary itself.

5 In spite of formidable difficulties in extracting and assaying the small amounts of oxytocin in the blood, Coch et al (1965) were able to demonstrate

increased plasma, oxytocic activity in blood samples drawn from the internal jugular veins of women during labour. The findings are in line with those on other mammalia. (Fitzpatrick, 1961, 1965).

It must be emphasised however, that the levels of oxytocin found in jugular vein specimens (500 μUnit/ml plasma or more) are much higher than the theoretical estimates of Saameli (1963) which are in the region of 3 μUnit/ml plasma. Again the high circulating levels of the hormone were only convincingly demonstrated in the second stage of labour. The role of oxytocin in initiating and maintaining first stage contractions is thus still questionable.

The release of oxytocin during labour may thus correspond to Ferguson reflex (1941) as shown in Fig. 4. Dilatation of the cervix in post-partal rabbits stimulates out-pouring of oxytocin from the neurohypophysis by afferent nervous stimuli carried up the spinal cord and relayed through the hypothalamus. The reflex can be abolished by spinal transection (T:7) and by cauterisation of the pituitary stalk.

2 OXYTOCIN AND LACTATION

Oxytocin causes contraction of the myoepithelial cells which surround the mammary alveoli and smaller ducts. Preformed milk secretions are thus forced into the larger

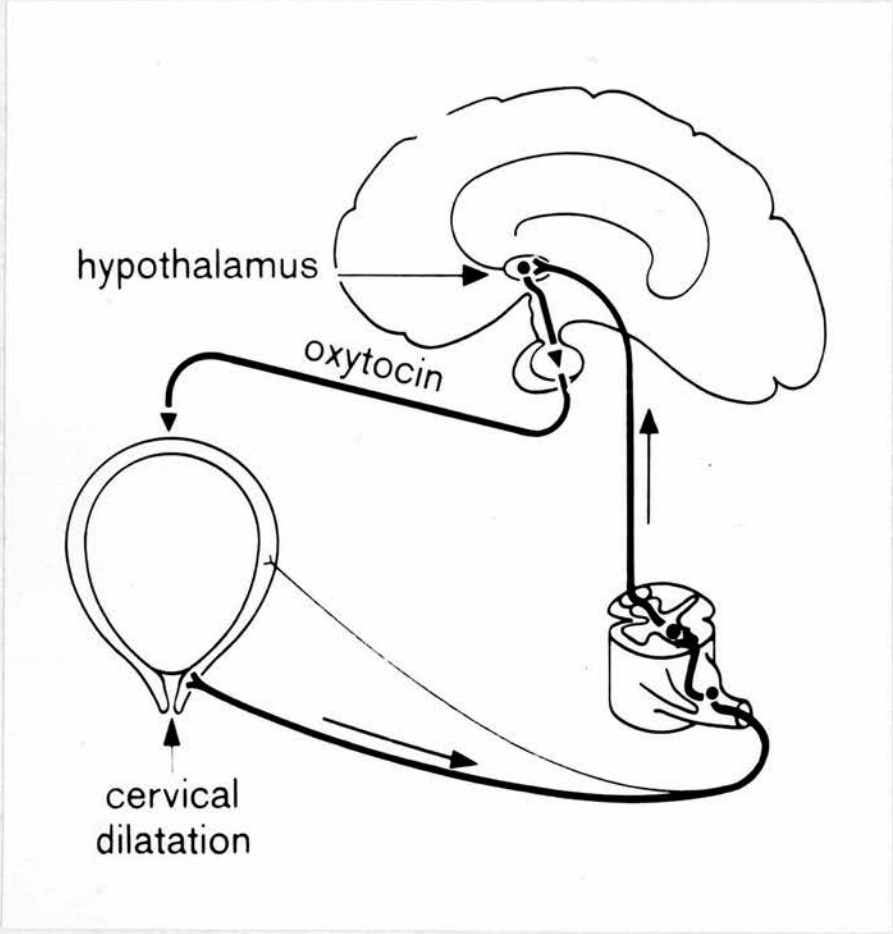


Figure 4 Reflex Release of Oxytocin as a Result of Cervical Dilatation (Ferguson Reflex).

lactiferous ducts and sinuses, raising the intra-mammary duct pressure and producing milk "let down" or "milk ejection".

Experimentally, contraction of the alveoli can be demonstrated following direct application of oxytocin in vitro (Linzell, 1959). Strips of lactating mammary tissue suspended in an organ bath contract on addition of oxytocin (Mendez-Bauer et al, 1960). In vivo, intravenous doses of oxytocin as small as 1 mU. cause an abrupt and transient rise in intramammary duct pressure in lactating rabbit (Van Dyke et al, 1955), and in the human female (Caldeyro-Barcia, 1960). Sustained infusion of oxytocin can produce rhythmic waves of increased pressure (Caldeyro-Barcia, 1960). The pattern of response simulates, but does not coincide with that of the uterus.

Physiological release of oxytocin during lactation in the human is evidenced by:

1 Milk-ejection from the contralateral breast during breast feeding.

2 Increased uterine activity during suckling (Cobo et al, 1965), and by mechanical stimulation of the nipples at the end of pregnancy, during labour or in the puerperium (Lorand and Asbot, 1952). Clinically it is also commonly observed that after-pains are commonly initiated or aggravated by breast feeding, presumably as a result of colicky uterine contractions induced

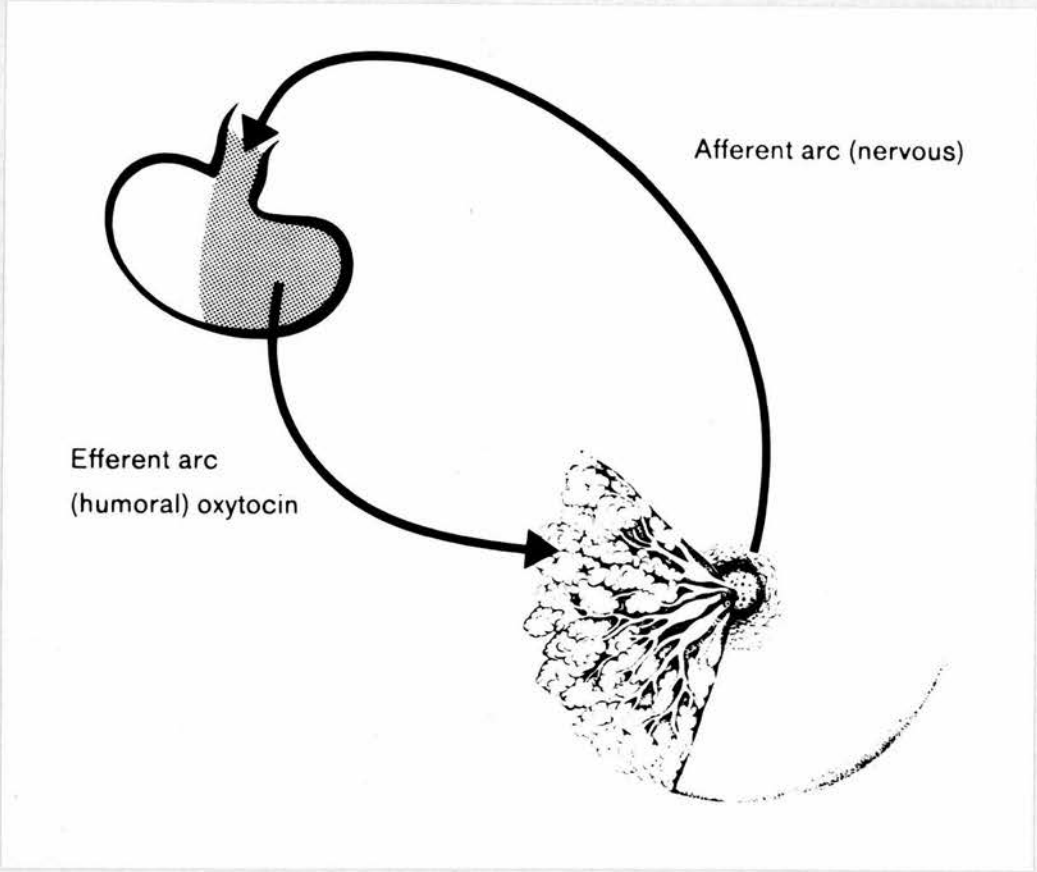


Figure 5 Reflex Release of Oxytocin as a Result of Mammary Stimulation (Milk-Ejection Reflex).

by circulating oxytocin.

The physiological stimulus for this milk "let down" reflex is probably mechanical stimulation of the nipple and areola during suckling (Fig. 5). The afferent side of the reflex is nervous; the sensory stimuli being relayed to the hypothalamic nuclei, which in turn induce release of oxytocin from the neurohypophysis. This is carried by the circulation to the breasts producing milk-ejection. In this way, previously secreted milk stored within the alveoli is made available for the suckling offspring (galactokinetic effect).

In addition to this direct milk-ejecting effect of oxytocin on the lactating breast, oxytocin appears to participate indirectly in promoting milk secretion by the alveoli (galactopoietic effect). Benson and Folley (1957) have suggested that oxytocin released during suckling stimulates release of prolactin (L.T.H.) from the anterior lobe of the pituitary gland. They reported that oxytocin injections could delay mammary involution in nursing rats after removal of their litter. No such effect was observed in hypophysectomised animals. This view finds support in the work of Desclin (1956) and Stunisky (1957) who were able to induce pseudopregnancy and formation of deciduomata in rats by oxytocin treatment. Other workers (e.g. McCann et al, 1960) were unable to confirm this finding. Thus at the moment, the galacto-poietic function of oxytocin remains a possibility for further investigation.

3 RENAL EFFECTS OF OXYTOCIN AND VASOPRESSIN AND THEIR POSSIBLE ROLE IN WATER AND ELECTROLYTE BALANCE OF NORMAL AND TOXAEMIC PREGNANCY

The effects of vasopressin and oxytocin in water and salt excretion by the kidney can be conveniently described under the following items:

a Urine Volume Regulation

b Saluretic Responses

c Effects on Renal Haemodynamics

Urine Volume Regulation

There is general agreement that the neurohypophysis is a main factor in the physiological regulation of the rate of urine excretion. Experimental injury to the neurohypophysis in the dog is followed by polyuria in the post-operative period (Schäfer, 1909). A similar finding has been reported in many other species. In man, the clinical syndrome of diabetes insipidus is invariably associated with lesions in the hypothalamic-neurohypophysial tracts.

The antidiuretic effect of vasopressin is best seen during a phase of diuresis following ingestion of a water load. In man, vasopressin in doses of 0.25 - 0.5 mU/min. is capable of inhibiting water diuresis (Theobald, 1955)[a].

The antidiuretic effect of vasopressin in mammals is often accompanied by elevation of the osmotic concentration in the urine above the plasma levels. There is, however, evidence that the production of concentrated

hyperosmotic urine is not directly dependent on the antidiuretic response (Berliner and Davidson, 1957; Smith,1952).

The site of action of the antidiuretic hormone in promoting water reabsorption from the glomerular filterate has been convincingly shown in the micropuncture experiments of Wirz (1957) to lie in the distal convoluted tubules and, possibly, the collecting tubules. The exact mechanism of action is not known. Morphological changes have been observed in the intercellular substance between the epithelial cells of the distal convoluted and collecting tubules following administration of vasopressin (Ginetzinsky, 1958).

The antidiuretic response to vasopressin is influenced by many factors including state of body hydration, the concomittent administration of oxytocin, and the state of electrolyte balance. This topic has been reviewed by Sawyer (1961).

Saluretic Effects

Neurohypophysial extracts enhance Na, K and chloride excretion in mammalian experimental animals. The occurrence and magnitude of saluresis depends on the peptides injected, the species and the preexisting conditions of salt and water loading. The effect can be produced by oxytocin as well as vasopressin in rats receiving NaCl infusion especially at low urine flow rates (Jacobson and Kellogg, 1956). When oxytocin saluresis occurs in an animal that is already excreting a moderately concentrated urine, the urine flow increases. This is a well defined

mechanism by which oxytocin can cause diuresis. In water loaded animals, however, oxytocin produces a clear antidiuretic response if sufficient doses are used. This antidiuretic effect is reproducible in man in whom saluretic responses have not been observed (Abdul-Karim and Assali, 1950).

Action on Renal Haemodynamics

Demunbrun et al (1954) reported depression of the renal function in the dog following experimental injury to the neurohypophysis resulting in diabetes insipidus; the effect was reversible by oxytocin administration. Brooks and Pickford (1960) were unable to demonstrate any depression of renal function with diabetes insipidus in dogs. However, they reported that in dogs moderate doses of oxytocin increase renal plasma flow, which could be inhibited by small doses of vasopressin. The role of oxytocin in the maintenance of renal function thus remains a possibility for further investigation.

The role of the neurohypophysis in water and electrolyte adjustments during normal pregnancy is not easy to define. In spite of definite water, Na and K retention in pregnancy, there is little evidence that the release of vasopressin, or the renal response to it are altered in normal pregnancy. Hawker (1952) reported that an antidiuretic substance (A.D.S.) was present in higher levels in sera of pregnant women, and postulated that it may account for some of the water retention during pregnancy. The assertion by several workers that water

diuresis is impaired in late pregnancy (McManus et al, 1934; Theobald, 1934a; Dieckmann, 1941) was challenged by Theobald (1955b), who reinvestigated the problem and came to the conclusion that the diuresis curves of the non-pregnant and normal pregnant subject are substantially the same. Govan and Garrey (1964) and Kerr (1965) emphasised the effect of posture on water diuresis in late pregnancy. This provides a possible explanation for the contradictory results of earlier works.

There is little information in the literature about the pattern of the renal response to vasopressin in pregnancy. Theobald (1934b) found that the antidiuretic response to a posterior pituitary extract was essentially the same in pregnant and non-pregnant women. Assali et al (1960) and Torres et al (1966) confirmed this finding, but reported differences in the effects of vasopressin injection on electrolyte excretion between pregnant and non-pregnant subjects.

Pickford (1952) considered the water retention of normal pregnancy as more likely to be secondary to salt retention produced by placental steroids.

In contrast to the scanty information about the neurohypophysial-kidney relationship in normal pregnancy, there is a vast body of literature on the possible role of the posterior pituitary gland in toxaemia of pregnancy. Earlier workers were impressed by the potent vasopressor and antidiuretic properties of posterior pituitary extracts which could account for the hypertension and oliguria of

pre-eclampsia and eclampsia. Hofbauer (1918, 1937) postulated a pituitary adrenal mechanism as the cause of pre-eclampsia.

Antidiuretic and vasopressor substances have been reported to be present in the sera of eclamptic women by Anselmino and Hoffmann (1930, 1931, 1932) and Mukherjee (1946). Many other workers, however, failed to demonstrate them (Byrom and Wilson, 1934; Hurwitz and Bullock, 1935; Krieger and Kilvington, 1946; and Levitt, 1936).

Similarly, there is a controversy on the presence of antidiuretic substances in the urine of eclamptic and pre-eclamptic patients and their nature (Teel and Reid, 1939; Ham, 1941; Ham and Landis, 1942; and Krieger et al, 1951). Paterson (1954) subjected the urine of normal and toxaemic patients to paper chromatography. He discovered in all cases of eclampsia and in a majority of cases of pregnancy hypertension, a urinary constituent which occupied a position on the chromatogram identical with that of the antidiuretic polypeptide described by Arneil and Wilson (1953). In a subsequent publication, Paterson (1961) confirmed his previous findings and identified this substance by biological and chemical studies to be allied to vasopressin.

The response of normal and toxaemic patients to the administration of vasopressin was studied by Dieckmann and Michel (1935, 1937); Shokaert and Lambillon (1937) and de Valera and Kellar (1938). There is general

agreement that pre-eclamptic patients react more markedly to injection of posterior pituitary extracts than normal patients, as evidenced by more pronounced hypertension and oliguria. Browne (1946) further concluded that this abnormal sensitivity to vasopressin was acquired after mid-pregnancy in patients who were destined to develop subsequent toxaemia.

Theobald (1955)$_6$ reported that the administration of vasopressin to hypertensive antenatal patients by intravenous infusion resulted only in a transient hypertensive response, and concluded that posterior pituitary hyperactivity could not account for the hypertension in pregnancy.

4 ADENOHYPOPHYSIAL EFFECTS OF POSTERIOR PITUITARY HORMONES

Popa and Fielding (1930) described a type of portal circulation between the neurohypophysis and adenohypophysis. The capillary plexuses of the median eminence (which is part of the neurohypophysis) collect into portal veins which are redistributed into the anterior lobe of the pituitary gland (adenohypophysis). Neurohypophysial products could thus reach the adenohypophysis in high concentration and may contribute an important link in the central nervous control over adenohypophysial secretion (Fig. 6).

Mention has been made of the possible effect of oxytocin on L.T.H. release. Similarly, vasopressin can

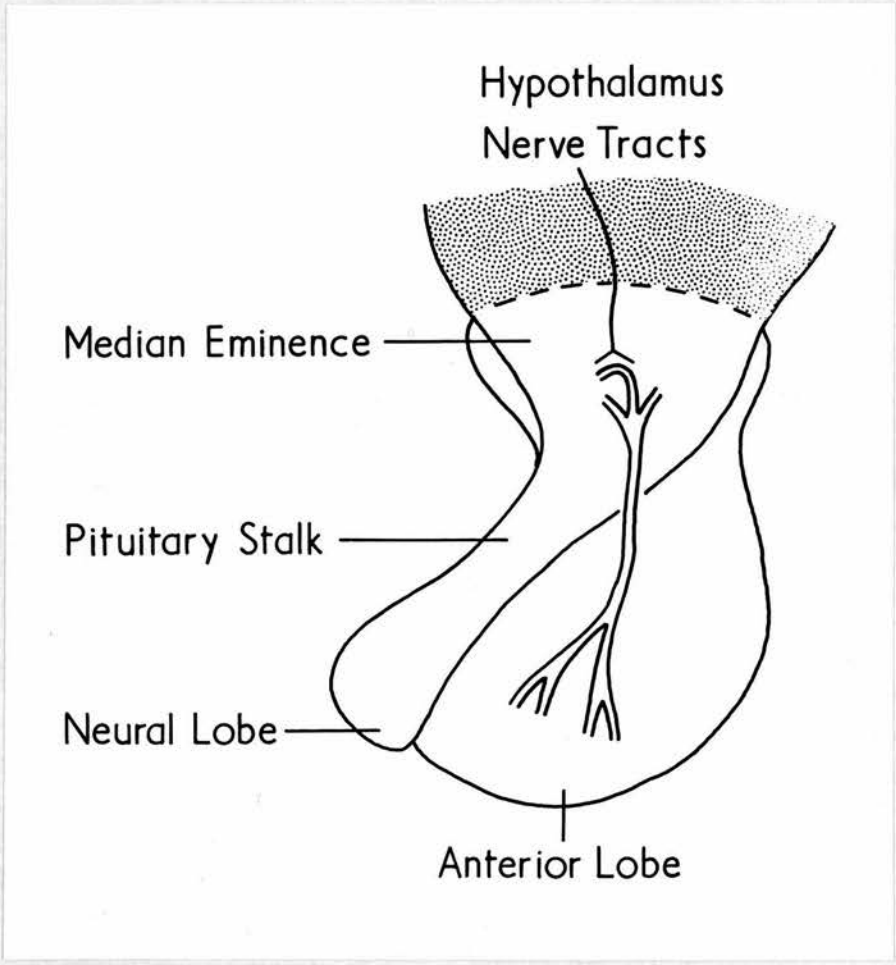


Figure 6 Diagram of a Sagittal Section through the Mammalian Hypothalamus and Pituitary Gland to Demonstrate the Portal Circulation between the Median Eminence (Neurohypophysis) and the Anterior Lobe of the Pituitary Gland.

elicit corticotrophin release (Hume, 1958; Guillemin et al, 1959). The doses required, however, are much higher than those producing maximum antidiuresis (Nichols and Guillemin, 1959). However, there is ample experimental evidence to indicate a regulating action of the neurohypophysis on anterior pituitary activity. Experiments involving secretion of the pituitary gland (Harris, 1955) and pituitary transplantation (Nikit and Everett, 1958) indicate that the anterior pituitary gland can only function normally in the presence of direct vascular connections to the neurohypophysis. Extracts of the median eminence and pituitary stalk from several mammals were shown to contain several "Trophin" releasing factors, including corticotrophin releasing factor (C.R.F.) (Saffran et al, 1955; Schally and Bowers, 1964), thyrotrophin releasing factor (T.R.F.), luteinising hormone releasing factor (L.R.F.) (Guillemin, 1964) and possibly follicular stimulating hormone and growth hormone releasing factors. The chemical structure of these factors has not been identified, but the available data suggested that they are relatively simple polypeptides.

5 OXYTOCIN AND SPERM TRANSPORT

The possible role of oxytocin in sperm transport has been reviewed by Fitzpatrick (1957), who concluded that the rapid ascent of spermatozoa in the female genital tract after mating is effected by uterine contractions

possibly due to release of oxytocin. Oxytocin has been reported to increase the rate of ascent of sperms in intact animals and in the isolated perfused uterus. Oxytocin release during mating is also evidenced by increased uterine activity and occurrence of milk ejection in animals and man. Increased blood levels have been reported in animals at the time of mating.

METABOLISM AND FATE OF OXYTOCIN AND VASOPRESSIN

Dale (1909) reported that intravenous injections of posterior pituitary extracts into cats resulted in the excretion of a pressor substance in urine. Larson (1935) found that the oxytocic as well as the pressor activity of posterior pituitary extracts was eliminated by the kidneys of anaesthetised cats and dogs, in amounts varying between 12.8 to 37.7% of the injected doses. Similar findings were reported in other species (Heller, 1937; Larson, 1939; Ingram et al, 1939). Gonzalez-Paniza et al (1961) reported that 20% of oxytocin administered by intravenous infusion in non-pregnant female subjects was recovered in the urine. In pregnant women, only 1% of the injected doses was excreted. Aroskar et al (1964) employing synthetic oxytocin with a tritium labelled leucine component, confirmed that following intravenous injection in rats, only part of the radioactivity (30 - 35%) is recovered in urine within a two hour period.

Many attempts at the elucidation of the fate of the unexcreted portion of oxytocin and vasopressin have been

made. Because of the great difficulties in assaying "endogenous" levels of the hormones, various workers investigated the fate of the hormones following injection of the hormones in comparatively large doses in order to achieve residual levels amenable to estimation by simple biological assay methods. These experiments constitute in vivo "clearance studies". Alternatively, oxytocin and vasopressin were incubated in vitro with various tissue extracts. Such studies constitute in vitro "inactivation" studies.

1 Clearance Studies

The studies of Ginsburg and Heller (1953) and Ginsburg and Smith (1959) indicate that exogenous vasopressin and oxytocin injected into rats are inactivated principally by the kidneys and liver. If these are excluded from the circulation, the rate of disappearance of the hormones is markedly decreased. Similar findings were reported in the dog (Lauson and Bacanegra, 1960) and rabbit (Chaudhury and Walker, 1959). There are no analogous data in human subjects, but there is no reason to doubt that the liver and kidneys are the main sites for clearance (and probably inactivation) of oxytocin and vasopressin from the circulation. It has been suggested that impaired water diuresis, oedema and ascites in patients with liver cirrhosis are due partly to decreased inactivation by the diseased liver. (Ralli et al, 1945).

The rate of clearance of injected oxytocin and vasopressin from the circulation of several laboratory animals

as well as the human has been investigated. "Half-life" values are interpolated from assay of the hormones on serial blood samples. The results obtained in various species are shown in Table 3. The results appear anomalous when compared to the duration of the effects of oxytocin and vasopressin on the target organs. In women near to term, the half life of the uterine effects of oxytocin infusions was calculated by Sica-Blanco and Sala (1961) as 15 minutes. Similarly, the antidiuretic effect of vasopressin on the kidney lasts 20 - 30 minutes or longer (de Wardener, 1957).

2 "In Vitro" Inactivation Studies

The finding that only a minor proportion of injected oxytocin and vasopressin can be recovered in a biologically active form has stimulated research on the possible inactivation of the hormones by various tissue extracts. Enzymatic inactivation of both polypeptides has been reported following their incubation with various tissue extracts. Such enzymes are commonly referred to as "tissue oxytocinase" and "tissue vasopressinase". As pointed out by Heller (1957) the specifity of these enzymes is not yet established, and it is hardly justifiable to give them such labels. All the more, since, in vitro, oxytocin and vasopressin can be inactivated by several known mammalian enzymes, e.g. chymotrypsin, spleen aminopeptidase and kidney hypertensinase (Croxatto et al, 1942). Vasopressin is also inactivated by trypsin (Lawler and Du Vigneaud, 1953).

TABLE 3

Peptide Injected	Species	Method of Assaying Residual Levels	Half-life in mins.	References
Vasopressin	Rat	Antidiuresis in Rats	0.8	Ginsburg and Heller, 1953
	Rabbit	"	3	Chaudhury and Walker, 1959
	Man	"	8	Schroder and Rott, 1959
Oxytocin	Rat	Oxytocic Effect on Isolated Rat's Uterus	1.6	Ginsburg and Smith, 1959
	Rabbit	"	3	Chaudhury and Walker, 1959
	Sheep	"	<1	Fitzpatrick, 1961
	Human Male	"	<1	Fitzpatrick, 1961
	Pregnant Female	Milk-ejecting effect	3	Gonzalez-Panizza, et al, 1961

Half-Life Clearance Values of Oxytocin and Vasopressin

Experiments with suspensions and homogenates of organs of various laboratory animals showed some loss of anti-diuretic, pressor or oxytocic activity in all instances (Heller and Urban, 1935; Larson, 1938; Birnie, 1953; Sawyer, 1954). The highest activity appeared to be in liver and kidney homogenates. Werle (1960) compared the responsible enzymes in various tissue extracts, revealing differences in pH activity ranges, and in their sensitivity to various inhibitor systems.

Ginsburg and Smith (1959) noted rapid in vivo clearance of oxytocin from the circulation in lactating rats, and suggested that significant elimination of oxytocin occurred in the mammary glands. Sawyer (1954) reported that homogenates of myometrium from pregnant or pseudo-pregnant rats destroyed oxytocin at rates comparable to the rates of inactivation by liver or kidneys. By contrast, in the rabbit uterus, the oxytocin inactivating enzyme(s) decreases during pregnancy and is minimal at term (Dicker and Whyley, 1960). No such activity could be demonstrated in the myometrium from several women near to term. Hawker (1956) reported inactivation of oxytocin and vasopressin following incubation with extracts of human placentae. Placentae of cases of pre-eclampsia had less activity per unit weight than those of normal pregnancy.

Hooper and Jessup (1959) and Hooper (1959) further studied "Oxytocinase" and "Vasopressinase" in extracts of human placentae. They reported that the two activities

are separable by ultracentrifugation. Oxytocinase remained in the supernatant, while vasopressinase was associated with mitochondrial and microsomal fractions. The two systems also had different pH optima and behaved differently towards organophosphorus inhibitors.

The roles of these various tissue oxytocin and vasopressin inactivating systems in the physiological metabolism of the hormones is not known. However, the work of Aroskar et al (1964) suggested that radioactive labelled oxytocin was widely distributed in the tissues within a short time after their intravenous injection. There it could possibly come under the effect of these various inactivating systems.

Rychlik (1964) reported a biochemical study on the mechanism of enzymatic degradation of oxytocin by a liver extract, employing paper chromatography, to identify the reaction products. He concluded that inactivation occurs in two steps. First, a reduction of the disulphide bond (position 1 - 6) occurred by a transhydrogenase enzyme. The reaction was greatly enhanced in presence of reduced glutathione and was probably reversible. In the second step, the open chain dihydro compound was affected by an aminopeptidase which did not attack the intact hormone itself. Fission occurred at the "cysteine" - tyrosine bond (1 - 2), and on longer incubation the rest of the molecule was systematically degraded.

PREGNANCY SERUM OXYTOCINASE

a Early Observations

In 1930, Fekete was first to observe that the serum of pregnant women is able to inactivate the oxytocic properties of posterior pituitary extracts. In a subsequent publication (1932) he asserted that this property of sera became more pronounced as pregnancy advanced. The loss of oxytocic activity was demonstrated on a preparation of isolated guinea pig uterus.

Shortly afterwards, Dietel (1933) reported the presence of a substance antagonistic to the antidiuretic effects of pituitary extracts in sera of pregnant women. SchocKaert and Lambillon in 1935 observed a similar loss of vasopressor activity of pituitary extracts incubated with pregnancy sera.

b Studies on Pregnancy Serum Oxytocinase by Biological Assay Methods

Werle and Effkemann (1941) employed the isolated rat uterus preparation to assay the residual oxytocin following incubation with pregnancy sera. They were the first to establish that the biological inactivation of oxytocin by late pregnancy sera is an enzymatic process. In 1941, Werle et al. found that the enzyme was only present in pregnancy sera, but was absent from sera of men and non-pregnant women. No activity was found in foetal cord blood, and thus they concluded that the enzyme did not cross the placental barrier. Moreover, Werle and co-

workers (1941) observed that the enzymatic activity in pregnancy sera was stable at room temperature, and was not reduced by storage at 4°C for one month. The activity was destroyed by boiling, but remained unaffected by dialysis against water for 24 hours. The maximal activity was found at a pH ranging from 6.5 to 7.5. Serum and plasma samples from the same subjects had identical activities.

Page (1946) made a similar study of the enzyme in pregnancy plasma and labelled it "Pitocinase". This name was later severely criticised by Heller (1957). Page (1946) reported that the kinetics of the enzymatic reaction under his conditions of incubation followed a first order reaction, and proposed a unit for enzyme activity based on the velocity constant. His finding, as regards stability and distribution of the enzyme in human pregnancy confirmed the work by Werle et al (1941). Page (1946) reported a progressive rise of the enzyme levels throughout pregnancy amounting to a thousandfold at term. Enzyme levels in cases of twins and intrauterine foetal death did not vary from the normal range. Pitocinase activity was found in high concentration in human placental extracts and in suspensions of haemolysed red blood cells, but was absent from sera of guinea pig, rabbit and rat. In 1947, Page suggested the use of pitocinase estimations as a pregnancy test and as a method for assessing the gestational age.

Werle et al (1950) studied the distribution of

oxytocinase in blood of various mammalian species. No enzymatic activity was found in the sera of horses, sheep, dogs, guinea pigs or rats, whether in the pregnant or non-pregnant state. Oxytocin-splitting enzymes were found only in the sera of pregnant women and apes. In 1951, Werle and Semm investigated the changes in enzyme levels throughout pregnancy. The enzyme was detectable by the 46th day of pregnancy, with rising levels in the second and third months of pregnancy. It remained at roughly the same levels from the third to the eighth month of gestation, and then began to rise again until at labour it reached a level sixty times higher than the levels at the end of the second month.

Subsequently (1956) Werle and Semm studied the effects of various inhibitors on pregnancy serum oxytocinase and reported inhibition by the metal-chelating agents E.D.T.A. and 8-Hydroxy-quinoline, which could be reversed by adding Co^{++}, Mn^{++} or Zn^{++}. They also investigated the distribution of oxytocinase in the various protein fractions of pregnancy sera. In vertical continuous electrophoresis the enzymatic activity was found in the cathode side of the albumin fraction and in the α_1 globulin fraction.

Werle (1960) compared the properties of oxytocin splitting enzymes systems in various tissue extracts with those of pregnancy serum oxytocinase, and pointed out the following differences:

a Pregnancy serum oxytocinase is more stable than "tissue oxytocinase".

b The serum enzyme is inhibited by metal-chelating agents, whereas the activity of the tissue enzymes is unaffected by those inhibitors. He thus concluded that serum and tissue oxytocinase are probably different enzyme systems.

Aragon (1948) and Woodbury et al (1948) investigated the enzyme levels in toxaemic patients, and were unable to demonstrate any significant differences from a normal control group. Both workers concluded that the hypersensitivity of pre-eclamptic and eclamptic patients to posterior pituitary hormones could not be attributed to diminished ability of the blood to inactivate them.

Dicker and Tyler (1956) investigated the enzyme levels for a possible drop at the time of onset of spontaneous labour which could be a causative factor in initiating normal labour at term. They reported that the ability of pregnancy sera to inactivate oxytocin decreased from the 28th week onwards, and could not be detected at term in the majority of cases. This finding was confirmed by Hilton and Johnson (1959). Dicker and Whyley (1959) reinvestigated the problem with a better controlled incubation technique and a 4-point biological assay on the isolated rat uterus. They found that the inactivation of oxytocin by pregnancy plasma reaches a maximal plateau in the last 4 weeks of pregnancy, with no drop during labour. The enzyme levels bore no relationship to the

occurrence of toxaemia in pregnancy or uterine inertia in labour.

Mendez-Bauer et al (1961) carried out a well controlled study of pregnancy plasma oxytocinase, assaying the residual oxytocin after incubation on the lactating mammary glands of rabbits (which is a more specific test organ for oxytocin). Plasma (9 volumes) was incubated with synthetic oxytocin (1 volume). The initial part of the reaction followed a first order pattern and the reciprocal of the half-time life was used as a measure of enzyme activity. They demonstrated a tenfold increase in the enzyme level by the end of gestation, with no drop before or at the time of onset of labour.

Parallel studies on pregnancy serum "vasopressinase" were carried out by Werle and Kalvelage (1941); Woodbury et al (1948); Dieckmann et al (1950); McCartney et al (1952); Croxatto et al (1953) and Barnes and Sawyer (1960). Following incubation of vasopressin with pregnancy sera, plasma or whole blood, the residual activity was assayed by its vasopressor or antidiuretic effects. The results indicated an increase of vasopressinase activity in pregnancy, with no difference between normal and toxaemic subjects. The enzyme was not detectable in foetal cord blood and disappeared slowly from the maternal circulation after delivery.

Dieckmann et al (1950) and Croxatto et al (1953) suggested that the same enzyme may be involved in the inactivation of both oxytocin and vasopressin, but gave

little direct evidence for this view. Werle (1960) on the other hand, suggested that pregnancy serum oxytocinase and vasopressinase are different enzyme systems. His argument is based on the finding that non-pregnancy sera also show a substantial vasopressinase activity. Furthermore, E.D.T.A. has no effect on pregnancy serum vasopressinase in contrast to its strong inhibitory effect on oxytocinase.

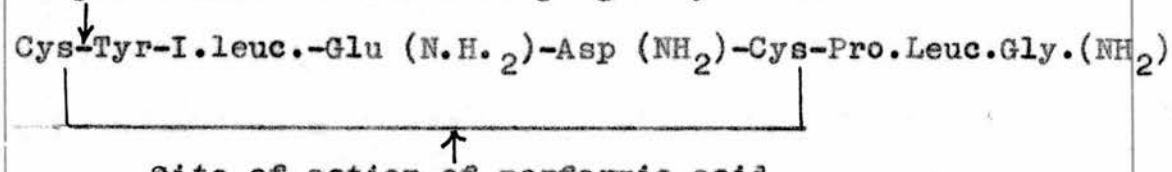
c Biochemical studies on Pregnancy Serum Oxytocinase

Biochemical studies on pregnancy serum oxytocinase only became possible after the molecular structure of the hormone was identified and pure synthetic preparations became available.

Tuppy and Nesvadba (1957) incubated oxytocin with serum of women in advanced pregnancy until the uterotonic effect of the hormone was lost. The incubation mixture was deproteinised with trichloracetic acid and the degradation product of oxytocin was oxidised with performic acid. Paper chromatography at this stage revealed the presence of free cysteic acid in addition to a polypeptide containing cysteic acid, tyrosine, isoleucine, glutamine, aspargine, proline, leucine and glycine. By comparison, oxidation of pure oxytocin yields a single polypeptide, with no free cysteic acid (Mueller et al, 1951). Tuppy and Nesvadba thus concluded pregnancy serum oxytocinase cleaves the oxytocin molecule at the peptide bond linking the tyrosine residue (position 2) to the terminal half-

cystine residue (position 1) which carries a free amino group.

Proposed site of action of pregnancy serum



The pregnancy serum enzyme thus appeared to be a proteolytic enzyme which acted as an aminopeptidase.

To test the validity of this concept, Tuppy and Nesvadba (1957) compared the aminopeptidase activities of pregnancy and non-pregnancy sera, employing the synthetic β-naphthylamide compounds of leucine, alanine and glycine. In presence of aminopeptidases, these compounds are split with the release of β-naphthylamine which can be measured colorimetrically. All these β-naphthylamide compounds were found to be readily split by non-pregnancy sera. In late pregnancy the splitting potency of the sera was enhanced by a factor of not more than 3.5. Considering the published data on oxytocinase which indicated its absence in non-pregnancy sera and a much greater rise during pregnancy, they concluded that the enzymes responsible for the cleavage of these 3 amino-acid β-naphthylamide compounds could not be identical with oxytocinase.

Tuppy and Nesvadba (1957) next investigated the possibility that oxytocinase is a specific aminopeptidase acting on substrates which, like oxytocin, possess amino-terminal half cystine residues. For this purpose, they synthesised and tested a new substrate: L-Cystine-di-β-

naphthylamide. When incubated with different sera this aminopeptidase substrate showed a pattern similar to oxytocin. Thus it was readily split by the sera of pregnant women, but only very slightly by sera of men and non-pregnant women. Thus they postulated that oxytocinase is a specific aminopeptidase which acts on compounds carrying half terminal amino groups, and that the synthetic substrate L-Cystine-di-β-naphthylamide, which carries a similar bond, can be employed for the enzyme assay.

To test the validity of this conclusion, Tuppy and Wintersberger (1961) carried out a stepwise purification of the enzyme, using retroplacental serum as a starting material. Purification involved ammonium sulphate precipitation, absorption on bentonite, and repeated column chromatography. The specific activity was determined at the various stages and an overall 4500 fold purification was achieved. The properties of the purifies sample were examined and conformed with the published data on oxytocinase assayed biologically. In addition, the purification products of the various steps were tested by Stoklaska and Wintersberger (1959) for their aminopeptidase activity as determined with the synthetic L-Cystine-di-β-naphthylamide substrate, and for their oxytocin inactivating potency as assayed by residual activity on the isolated rat uterus. Throughout the process of fractionation, the two activities proved inseparable; they retained the same unchanged ratio, and showed identical pattern of rise in specific activity.

Aim of the Present Study

This study is concerned with throwing light on the following aspects of the problem:

1 Assessment of the primary site of action of the pregnancy serum enzyme(s) on the oxytocin and vasopressin molecules. i.e. of the identity of pregnancy serum oxytocinase and vasopressinase.

2 Evaluation of the chemical methods for estimation of pregnancy serum oxytocinase.

3 Study of the distribution of the enzyme in the sera of women in normal and abnormal pregnancy.

4 Investigation of the site of origin of pregnancy serum oxytocinase and its possible physiological role.

EXPERIMENTAL PART

CHAPTER I

ANALYTICAL DATA

1 Reagents

Unless otherwise stated, all chemicals used in this study were British Drug House products of the Analytical Reagent Grade.

2 Buffers

The following buffers were employed -

a Phosphate Buffers

Phosphate buffers were prepared from crystalline Sodium Monohydrogen Phosphate ($Na_2H.PO_4.12H_2O$, Mol. Wt. 358.22) and Sodium Dihydrogen Phosphate ($NaH_2PO_4.2H_2O$ Mol. Wt. 156.03). Stock solutions of both salts were prepared in an ionic strength 0.2 M. The final buffers were obtained by mixing the two solutions in the following proportions:

	Na_2HPO_4	NaH_2PO_4
pH 7.4 M 0.2	81.0	19.0
pH 8.0 M 0.2	94.7	5.3

b Veronal Buffer (pH 7.9) M 0.046

68.9 ml. of 0.1N sodium barbitone (20.6 gm. sodium diethyl-barbiturate per litre of boiled carbon-dioxide free distilled water) is mixed with 31.1 ml. of o.1 N HCl and 50 ml. distilled water.

c Tris. Buffer (pH 7.9) M 0.1

A stock solution of 0.4 M Tris. (Hydroxymethyl) amino-methane (Mol. Wt. 121.14) was prepared

(48.46 gm. per litre of distilled water) . The buffer was then obtained by mixing 25 ml. of this solution with 32.5 ml. 0.2 N HCl and diluted to 100 ml. with distilled water.

The pH of all buffers was checked by the glass electrode and the solutions were stored at 4°C.

3 pH Measurements

Whenever possible, pH measurements were performed on a Pye-Cambridge glass electrode pH meter. Occasionally, with small volumes, approximate readings were obtained by using B.D.H. pH paper strips.

4 Spectrophotometric and Fluorimetric Estimations

All reactions which required fluorimetric or spectrophotometric estimations were carried out in duplicate tubes with a blank and a standard for each experiment.

Spectrophotometric estimations were carried out in a Unicam Sp. 600 using rectangular 10 m.m. cuvettes. The blanks (or zero time controls) were set to zero and the readings for the standard and unknown solutions were registered as optical densities (O.D.).

Fluorimetric estimations were done in a Locarte single sided Fluorimeter MK 4, using cylindrical cuvettes 10 m.m. in diameter. Blanks were set to zero and the high standard was set at 100. The fluorescence of the unknown solutions was read as percentage of the high standard after substracting the reading for the zero time control.

5 Statistical Analysis

Arithmatic means were compared by Student t test at a minimal significance level of P 0.05.

Standard deviation in small samples (< 10) was computed from the ranges (Gaddum, 1953).

Statistical analysis of the biological 4-point assays of oxytocin on rat uterus was carried out by the simplified methods of Noel (1945). The following symbols were employed:

N	Number of sets of observations
S_1 and S_2	Effects of low and high doses of the standard
U_1 and U_2	Effects of low and high doses of the unknown
I	Log. ratio of doses
M	Log. ratio U/S
S	Summation sign
s	Standard deviation of a single observation
b	Estimate of slope
R	% potency U/S
λ	Index of precision
λR	Standard error of R

6 Collection and Storage of Blood Samples

Maternal blood samples were obtained from the arm veins. Haemolysis was avoided by emptying the syringe slowly and refraining from forcibly expelling any residual

froth. Heparinised plasma was used with the biological method for enzyme assay, otherwise sera were employed. In all cases serum (or plasma) was separated by spinning the blood sample at 2000 r.p.m. for 10 minutes within two hours from collection. The serum (or plasma) was decanted and stored at $4^{o}C$. Enzymatic estimations were always carried out within 48 hours from collection.

Foetal blood samples were obtained from full time infants, at the time of delivery, by milking the umbilical cord. No attempt was made to secure venous and arterial blood samples separately.

Uterine vein blood samples were obtained from patients undergoing lower segment Caesarean Section near to term. After the peritoneum of the utero-vesical pouch was opened, the main stem of the uterine vein was exposed at the side of the uterus, and a blood sample was aspirated.

Blood samples from small animals (rat and mouse) were obtained by decapitation. Blood samples from cow, sheep and pigs were collected by venipuncture.

E X P E R I M E N T A L

P A R T

C H A P T E R II

A COMPARATIVE STUDY OF PREGNANCY PLASMA AND PLACENTAL OXYTOCINASE ACTIVITY BY A BIOLOGICAL ASSAY METHOD

Although a placental origin of pregnancy plasma oxytocinase has been suggested by Page (1946), Carballo and Mendez-Bauer (1957) and Semm (1961), inhibitor studies seem to indicate differences in the properties of the two enzyme systems. Werle and Semm (1956) reported that the serum enzyme is inhibited by metal chelating agents such as E.D.T.A. Placental oxytocinase on the other hand, was reported to be unaffected by E.D.T.A. (Hooper, 1959). Hooper (1959) however, stated that it is possible that inhibitors ineffective under his experimental conditions may be effective at a different pH or with a longer contact time.

As the experimental conditions differed in the two reports, this study was undertaken to investigate the inhibitor spectra of the plasma and placental oxytocinase activities under similar conditions.

BIOLOGICAL METHOD FOR ASSAY OF OXYTOCINASE

The essential part of this method is the biological assay of residual oxytocin following incubation with placental extracts or pregnancy plasma under suitable conditions.

For this purpose, isolated rat uterus was employed according to the method of Holton (1948). Although more sensitive methods are available (Table 1) for the assay of oxytocin, this method is sensitive enough for the

present study. It is also quick and has good precision.

(I) Assay of Oxytocin on the Isolated Rat Uterus

For this purpose mature female rats weighing 160-200 gm. were selected and prepared by a subcutaneous injection of a dose of 20 µgm. of stilboestrol in arachis oil 16 - 24 hours before the actual assay. This dose of stilboestrol induced oestrus in the rat with attendant changes in the uterine horns which became oedematous, hypertrophic and more sensitive to oxytocin (Gaddum and Hameed, 1954). It has also been shown that in proestrus and oestrus the rat uterus has least spontaneous activity, and this is a desirable feature in oxytocin assay (Flatters, 1954). Although suitable animals can equally be selected by daily vaginal smears (Flatters, 1954) pretreatment with an oestrogenic substance obviated the need for maintaining a large colony of animals.

The chosen animal was killed by a sharp jerk to the neck, producing dislocation of the cervical spine. The two uterine horns were dissected out. One horn was used for the actual assay. The other horn was stored at $4^{o}C$ in the solution described by De Jalon and De Jalon (1945) and could be used satisfactorily within 1 to 2 days.

The cervical part of the horn was used for the assay as it has less spontaneous activity than the ovarian end (Werle and Semm, 1951). About 2 cm. length was secured from the cervical end of the horn and suspended in the tissue chamber (2 ml. capacity) of an organ bath. The lower end of the horn was fixed to a metal hook, and the

upper end was attached by thread to the short arm of a frontal writing level. The tissue chamber was lowered into the organ bath and was filled up with a modified Locke's solution of the following composition:

NaCl	9 gm	$MgCl_2$	0.005 gm
KCl	0.42 gm	$NaHCO_3$	0.5 gm
$CaCl_2$	0.06 gm	Glucose	0.5 gm
Glass distilled water	1000 ml		

The ingredients were prepared in concentrated stock solutions and stored at 4°C. The final solution was freshly prepared every 2 - 3 days, in one litre amounts. Compared to Locke's solution, this medium has a reduced calcium and glucose content ($\frac{1}{4}$ and $\frac{1}{2}$ respectively). As reported by De Jalon and De Jalon (1945), this medium reduces the spontaneous activity of the rat's uterus. The bathing fluid was led into the bottom of the tissue chamber through a glass coil immersed in the surrounding water bath, the temperature of which was maintained at 29°C ($\pm$ 0.5) by means of a thermostat. The tissue chamber was aerated by a uniform current of a mixture of 5% CO_2 in 95% oxygen bubbled into the base of the chamber through a fine hypodermic needle.

Recording of uterine contractions was obtained by a frontal writing lever on a smoked drum. A load of 1.2 - 1.5 gm. was applied on the uterine side of the lever, which had a magnification of 1:6.

Following suspension of the horn in the organ bath, the preparation was left to equilibrate for half an hour

with frequent changing of the bathing fluid, before the actual assay was begun.

Doses of standard oxytocin solution were then added to the bath by a blow-out pipette to determine the range of sensitivity of the preparation. Contractions usually occurred within 30 seconds after an effective dose. After the peak of the contraction had been recorded and the lever was steadily going down, the tissue chamber was drained and washed out twice with bathing fluid and finally refilled. The subsequent dose was added exactly 3 minutes after return of the lever to the base line, and the whole procedure was repeated. Once the response of the preparation to the standard oxytocin solution became stabilised, assay of the oxytocin potency of the unknown solution was carried out by one of two methods.

a - Bracketing

Two dose levels of standard were given before and after two dose levels of the unknown, the unknown being diluted until responses were approximately equal to those of the standard. This method gave an approximate estimate of the oxytocin content of the unknown, but no reliable estimate of the error of the assay. It was used in the preliminary studies on oxytocinase.

b - Four-point assay method (Schild, 1942)

Two doses of the standard solution (S_1 and S_2) were found out by trial and error which resulted in contractions approximately 30% and 80% of the maximum. The ratio between the two doses varied in different preparations, but usually

lay between 1:2 and 3:4. At the same time, two doses of the unknown solution (U_1 and U_2) were chosen to give contractions of approximately the same heights as the standard. For the final statistical analysis the ratio U_1/U_2 should be the same as S_1/S_2. The four selected doses were subsequently administered in a random order, the whole procedure being repeated 3 to 5 times, using a different random order of doses each time. The total assay thus comprised 12 - 20 contractions, and was usually completed in 1½ to 2½ hours. A suitable preparation could usually be used for two successive assays. The smoked paper was then varnished and hung up to dry.

This method allowed a more accurate determination of the strength of the unknown solution, as well as an estimate of the range of error.

Fig. 7 represents a complete 4-point assay.

The statistical treatment of the data was performed according to the simplified method described by Noel (1945) and Gaddum and Lembeck (1945). The data are shown for the same experiment in Table 4.

(II) Conditions for Incubation

In order to compare the effects of various inhibitors on pregnancy plasma and placental oxytocinase, it was necessary to investigate certain aspects of the enzyme kinetics and establish standard conditions for incubation suitable for measuring inhibition. For this purpose, the

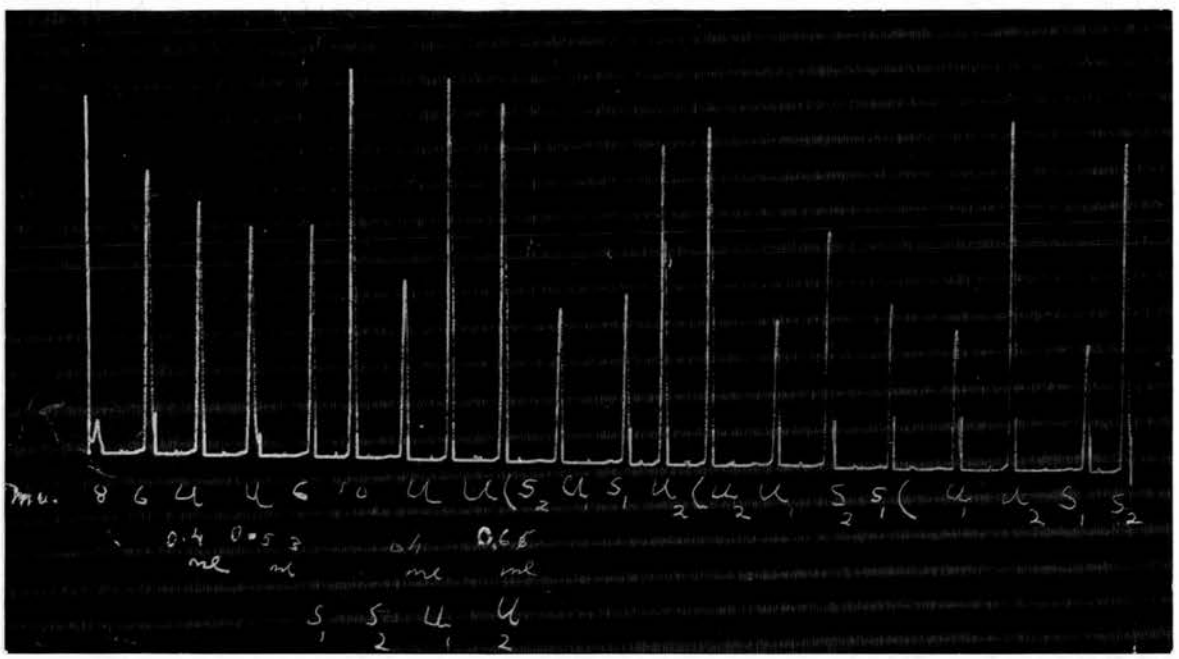


Figure 7 4-Point Assay of Oxytocin on the Isolated Rat Uterus. (Photograph Unmagnified).

TABLE 4

Calculations for the Assay shown in Fig. 6

N =	4 Sets of Results	1	2	3	4
S_1	Effects of Standard (Height of Contractions	30	22	21	17
S_2	in m.m.)	51	47	30	43
U_1	Effects of Unknown	24	20	18	18
U_2		50	42	44	45
D_1	$U_2 - S_2$	- 1	- 5	+14	2
D_2	$U_1 - S_1$	- 6	- 2	- 3	- 1
D_3	$U_2 - U_1$	26	22	26	27
D_4	$S_2 - S_1$	21	25	9	26
Y_1	$D_1 + D_2$	- 7	- 7	11	3
Y_2	$D_3 + D_4$	47	47	35	53
Y_3	$D_1 - D_2$	6	- 3	17	1

T_1	$S(Y_1)$	Sample Difference	0
T_2	$S(Y_2)$	Dose Difference	182
T_3	$S(Y_3)$	Slope Difference	21
I	Log. Ratio of Doses	Log 10/6	0.2218
M	$T_1 I/T_2$	Log Potency U/S	0.0
R	100 Anti Log. M	Potency of U (% of S)	100
b	$T_2/2$ IN	Slope	102.6
A	$S(Y_1)^2 + S(Y_2)^2 + S(Y_3)^2$		90.5
B	$T_1^2 + T_2^2 + T_3^2$		33565
S	$\sqrt{(A-B/N)/12(N-I)}$	Standard deviation of single observation	5.07
λ	s/b	Standard deviation of log dose	0.049
λM	$\sqrt{(1+T_1^2/T_2^2)/N}$	Standard deviation of M	0.025
λR	2.3 x λM x R	Standard deviation of R	5.8
t	$T_3/2S\sqrt{N}$	Test for parallelism	1.05
			Non Significant at 5% level

Oxytocin content of unknown is 100% ($\pm$ 5.8) that of standard.
The unknown therefore contains 6 or 15 m.U/ml. ($\pm$ 0.9)

enzymatic activity in late pregnancy plasma or placental extract was estimated by incubation with synthetic oxytocin (Syntocinon, Sandoz) in presence of phosphate buffer (pH 7.4, ionic strength 0.2 M). The concentration of oxytocin was standardised at 1 Unit/ml. of the incubation mixture, and the following aspects of the enzyme kinetics were investigated.

1 Time course of enzymatic inactivation (Progress Curve)

2 Effect of varying enzyme concentrations

3 Stability of the enzymes

(1) Progress Curve

Two mls of plasma from subjects 36 to 40 weeks pregnant were incubated with 20 U of oxytocin (2 mls.), and the final volume was brought up to 20 mls. with phosphate buffer. Immediately after mixing, a 5 ml. sample was pipetted off into a separate tube and dipped into a boiling water bath for 5 minutes and served as zero time control. The remaining part of the mixture was incubated in a water bath at 37°C. Specimens were withdrawn at various time intervals and dipped into a boiling water bath for 5 minutes to stop the enzymatic reaction. After cooling, all specimens were filtered. The residual oxytocin was assayed by the bracketing technique on isolated rat uterus, using the zero time control specimens as a standard.

The experiment was repeated with 3 different plasma samples from women in late pregnancy. The results are

shown in Fig. 8. The enzymatic inactivation appears to follow a first order reaction. This finding is in agreement with the reports of Werle et al (1941); Page (1946); and Dicker and Whyley (1959). Approximately 30 - 50% of oxytocin in the incubation mixture was inactivated in 10 minutes, and 75 - 90% in 60 minutes. A similar finding has been reported by Dicker and Whyley (1959). Accordingly, under these conditions of incubation, the relationship between Log percentage residual oxytocin and time (in minutes) is approximately linear with a negative slope (Fig. 9).

(2) Effect of Varying the Enzyme Concentration

(a) Plasma Oxytocinase

Varying volumes of late pregnancy plasma (0.1 - 2 ml.) were incubated for 60 minutes with 10 U oxytocin in a final volume of 10 ml. phosphate buffer. The reaction was then stopped by heat and the residual oxytocin assayed by the bracketing technique. The results obtained with 3 different plasma specimens are shown in Table 5. In Fig. 10 the plasma volume in the incubation mixture is plotted against Log percentage residual oxytocin. The relationship is approximately linear with a negative slope within the range 0.1 to 1 ml. plasma/10 ml. incubation mixture. Employing a concentration of 1 ml. of late pregnancy plasma in 10 ml. incubation mixture, differences in Log percentage residual oxytocin after one hour incubation could thus be accepted as a measure of enzymatic activity.

TABLE 5

% Residual Oxytocin	ml. Plasma/10 ml. Incubation Mixture						
	0.2	0.4	0.6	0.8	1.0	1.5	2.0
	48	40	22.5	-	10	<2	-
	64	60	30	-	12.5	<5	-
	80	50	-	40	25	9	5

Percentage Residual Oxytocin after 60 minutes Incubation with Various Volumes of 3 Different Plasma Samples of Subjects at 36-40 Weeks of Pregnancy

TABLE 6

% Residual Oxytocin	ml. Placental Extract/10 ml. Incubation Mixture								
	0.1	0.2	0.3	0.4	0.5	0.75	1.0	1.5	2
	55	25	10	6	2	<1	<1	-	<1
	60	32	15	7	6	5	<1	<1	-
	74	58	32	22	10	6	<1	<1	-

Percentage Residual Oxytocin after 60 minutes Incubation with Various Volumes of 3 Different Placental Extracts in Saline 1:1 (wt/v.)

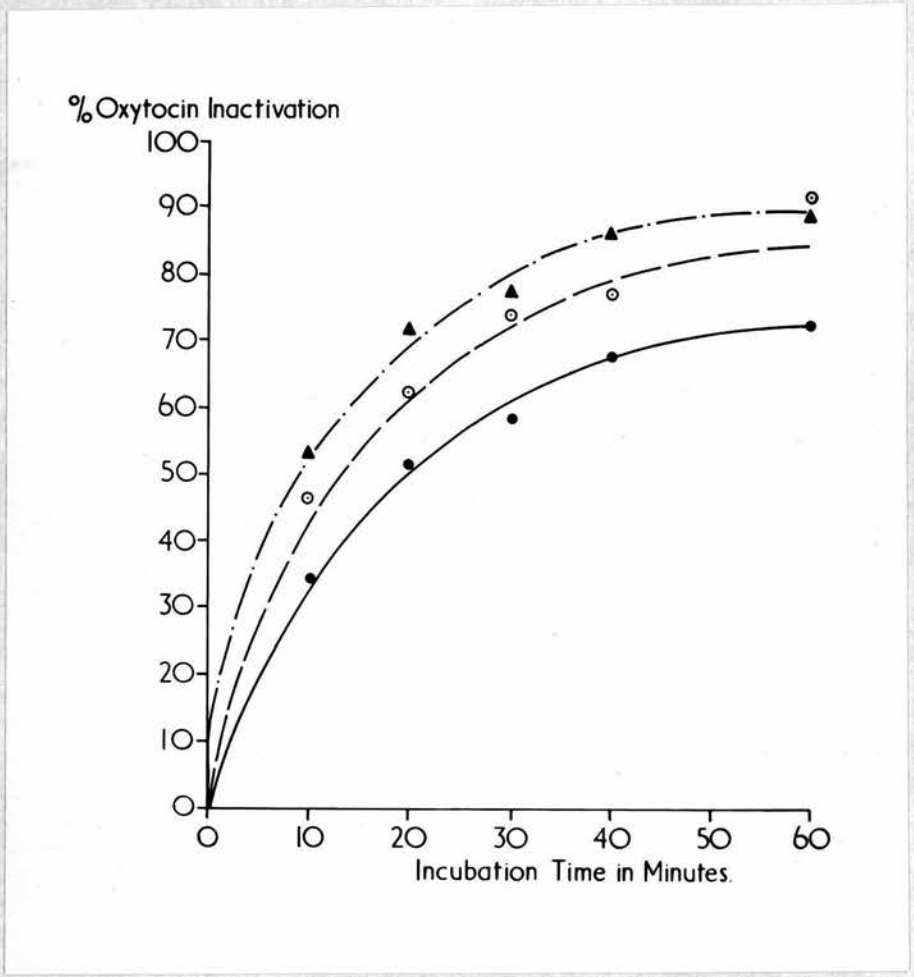


Figure 8 Time Course Relationship of Oxytocin Inactivation by Plasma of Women in Late Pregnancy.

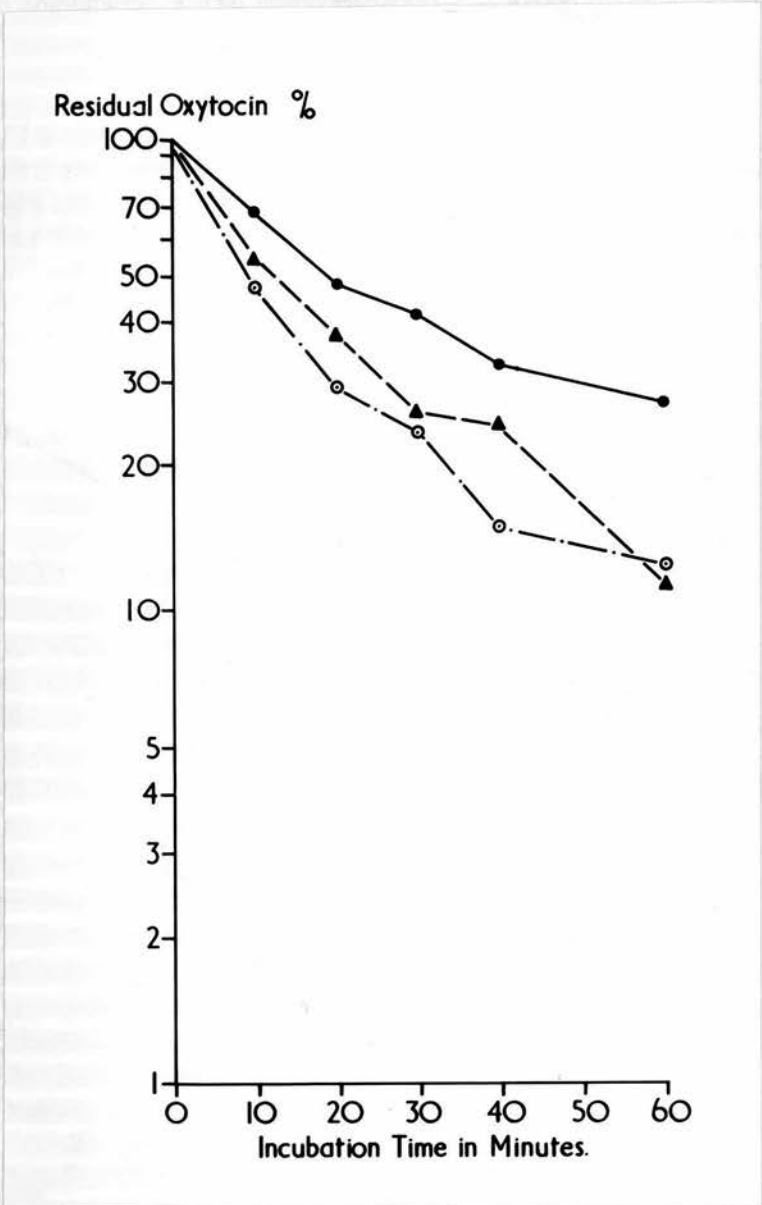


Figure 9 Progress Curve of Oxytocin Inactivation by Plasma of Women in Late Pregnancy. Logarithmic Scale on the Ordinate.

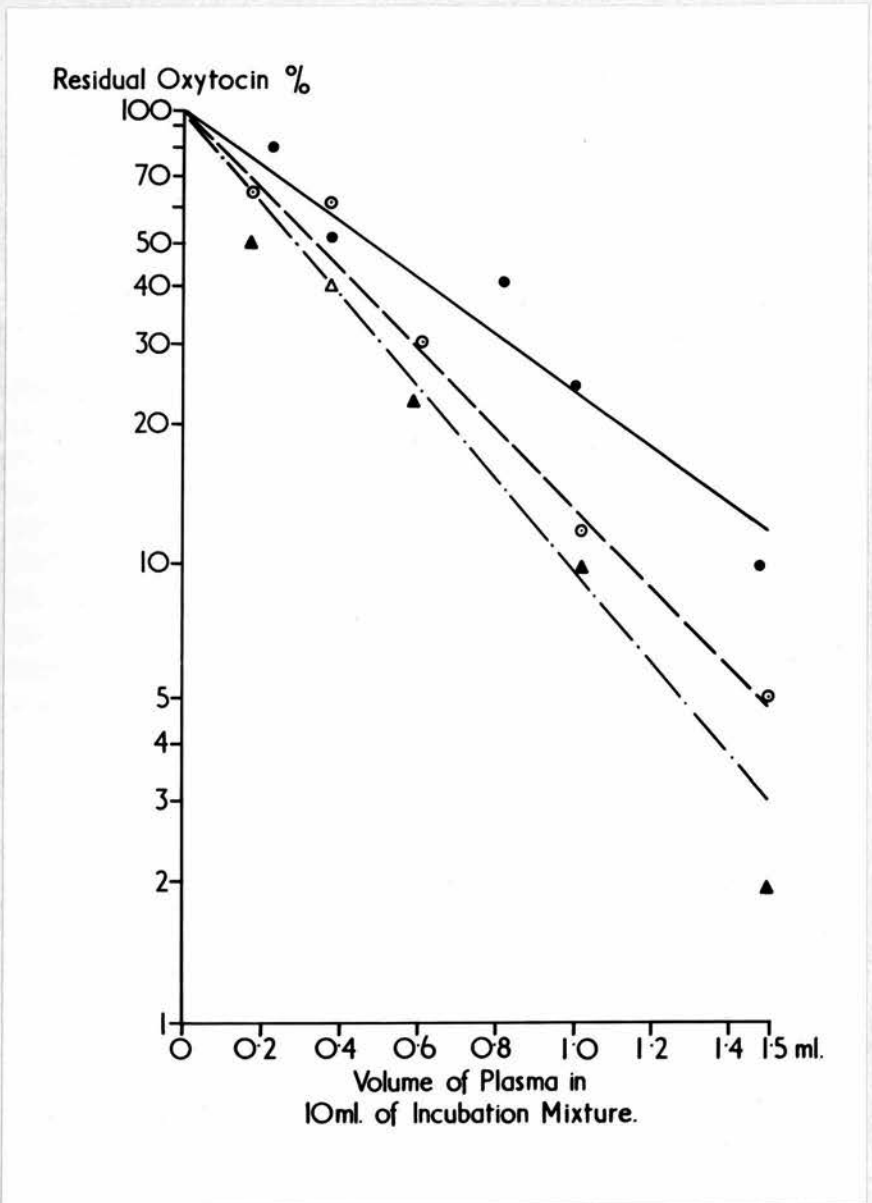


Figure 10 Relationship between Changes in Plasma Concentration and Oxytocinase Activity (Expressed as Log. Percentage Residual Oxytocin after Incubation for 60 minutes).

(b) Placental Oxytocinase

Extracts of full term placentae were prepared according to the method described by Hawker (1956). Fresh placentae from cases of normal delivery were extracted within 12 hours. The membranes were stripped off and all adherent clots were washed off under a current of tap water. Pieces of tissue approximately 5 m.m. thick were sliced off the maternal surface and floated for 10 minutes in a basin containing tap water to free them from blood. The tissue slices were then blotted between filter paper. Twenty grams of tissue were then weighed and mixed with 20 ml. of 0.9% NaCl solution and a suitable quantity of silver sand in a porcelain mortar, and then thoroughly homogenised for 20 minutes by the pestle. The homogenate was then centrifuged at 3000 r.p.m. for 15 minutes. The supernatant was decanted and employed as a source of placental oxytocinase.

Various volumes of the extract (0.1 - 2 ml.) were incubated with oxytocin (10 U), the final volume being brought to 10 ml. with phosphate buffer. A zero time specimen was secured and deproteinised by heat. At the end of 60 minutes incubation, the test mixture was treated similarly. The solutions were cooled and filtered. The residual oxytocin in the test mixture was assayed, using the zero time control as standard. The results with 3 different extracts are shown in Table 6 and Fig. 11.

The enzymatic activity of 0.4 to 0.5 ml. of the placental extracts was found approximately equivalent to

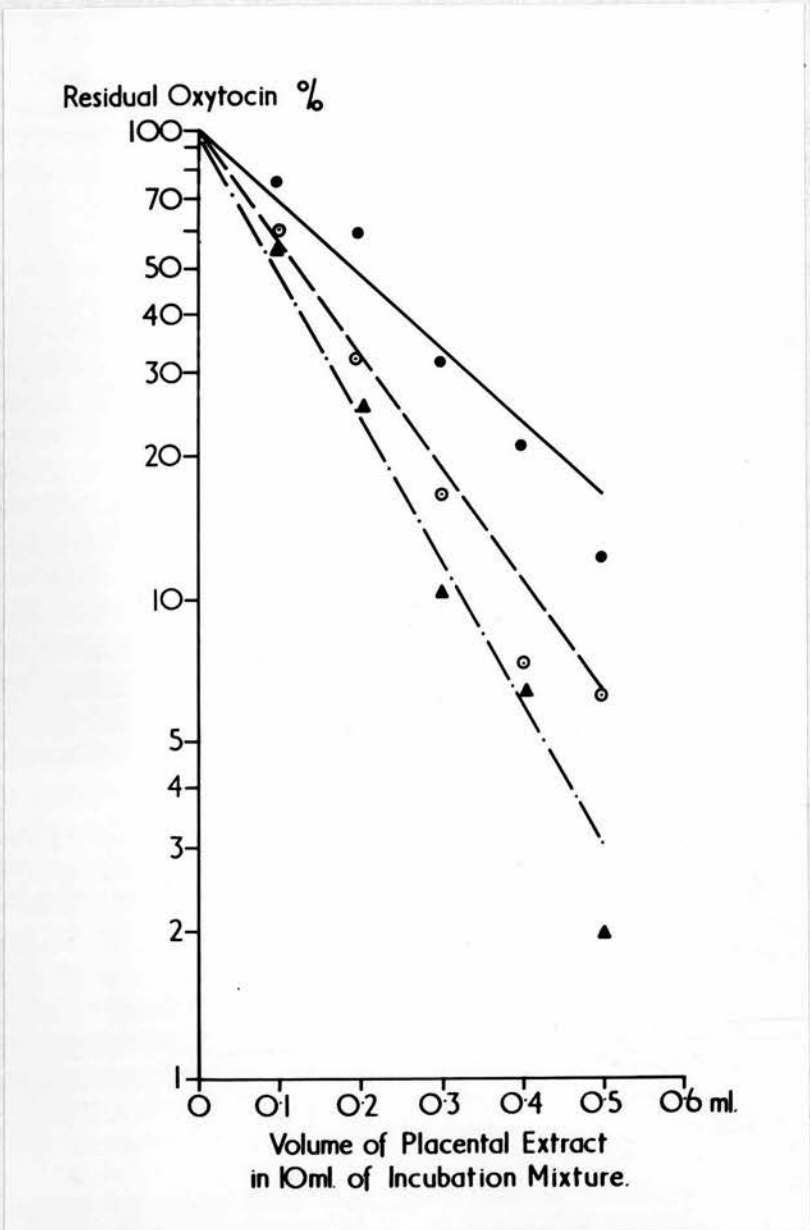


Figure 11 Relationship between Changes in Placental Extract Concentration and Oxytocinase Activity (Expressed as Log. Percentage Residual Oxytocin after Incubation for 60 Minutes).

one ml. of late pregnancy plasma, producing inactivation of 80 to 90% of oxytocin in 60 minutes under the present experimental conditions. In the range of 0.1 to 0.4 ml. of placental extract per 10 ml. of incubation mixture, Log percentage residual oxytocin showed a linear negative correlation with the concentration of placental extract in the mixture (with a negative slope) (Fig. 11).

(3) Stability of the Enzymes

Plasma samples (one ml.) were assayed for oxytocinase activity as before on the day of collection. The remaining part of the plasma sample was stored at 4°C. Estimations were repeated 24 hours and 48 hours later. Comparable inactivation of oxytocin was obtained with all 3 specimens indicating no loss of activity with storage. The experiment was repeated with two different placental extracts (using 0.4 ml. of extract) with similar results.

(III) Inhibitor Studies

For a comparative study of the effect of various enzyme inhibitors on oxytocinase activity in pregnancy plasma and placental extracts, the conditions for incubation were standardised as follows:

Plasma from women 36-40 weeks pregnant	1.0 ml.
(or placental extract)	(0.4 ml.)
Oxytocin (Syntocinin)	10 Units
Phosphate Buffer (M 0.2, pH 7.4)	ad. 10 ml.

A zero time sample (4 ml.) was pipetted off into a

separate tube after mixing, and deproteinised by dipping in a boiling water bath for 5 minutes. The remaining part of the mixture was incubated for one hour at 37°C (± 0.5) in a water bath, and then similarly deproteinised by heat. For inhibition experiments, the plasma (or placental extract) was incubated with an equal volume of the inhibitor solution for 15 minutes at 37°C. All inhibitors were added in aqueous solution containing 2×10^{-3}M, thus securing a final concentration of 10^{-3}M. The only exception was E.D.T.A. (Ethylene Diamine-Tetra-Acetate), where a concentration of 10^{-2}M was employed as in Werle's work (1960).

Following preliminary incubation with an inhibitor, the other ingredients of the incubation mixture (oxytocin 10 U and phosphate buffer, ad 10 ml.) were added. A zero time sample was pipetted off and the experiment proceeded exactly as with the control tubes.

The effect of the following inhibitors was investigated:

(1) Heavy Metals

Cu^{++} 10^{-3}M (as $CuSO_4 \cdot 5H_2O$)

Zn^{++} 10^{-3}M (as $ZnSO_4 \cdot 7H_2O$)

Ag^{+} 10^{-3}M (as $AgNO_3$)

Fe^{++} 10^{-3}M (as $FeSO_4$)

(ii) Metal Chelating Agents

E.D.T.A. 10^{-2}M (disodium salt)

8-Hydroxyquinoline 10^{-3}M

(iii) Sulphydryl Inhibitors

Iodacetamide 10^{-3}M

p. Chlormercuric benzoate (sodium salt) 10^{-3}M.

All solutions were cooled to room temperature and filtered before assaying the residual oxytocin content by the 4-point technique against the corresponding zero time sample. In addition, the oxytocin content of the zero time samples was checked against a standard of oxytocin diluted in phosphate buffer in 12 experiments (including one experiment with each inhibitor).

The effects of various inhibitors were evaluated against the percentage residual oxytocin in the control tube. As previously established, under the present conditions of incubation, the latter reading bears a negative linear relationship to the enzyme activity when plotted on a semilogarithmic scale. The percentage inhibition was then calculated from the equation

$$\frac{\text{Log a} - \text{Log b}}{\text{Log 100} - \text{Log b}} \times 100$$

Where a is the percentage residual oxytocin after incubation in presence of a specific inhibitor, and b is percentage residual oxytocin in a parallel control experiment with the same plasma. A similar method for calculation was employed by Hooper (1959), who, however, used the same placental oxytocinase preparation in all experiments, and compared the results of inhibition with a standard curve for his preparation.

RESULTS

(1) Observations on the Biological Assay of Oxytocin on the Isolated Rat Uterus

In the course of this study, the uteri from 116 rats were employed. Of these, 34 proved to be unsuitable. The main difficulties encountered were -

i Persistent and excessive spontaneous motility. Spontaneous contractions were invariably noticed when the uterine horn was initially suspended in the bath, but usually settled down after a period of half to one hour. Occasionally, however, activity persisted, making the preparation unreliable for the assay of oxytocin. In some cases, excessive motility was overcome by reducing the temperature of the organ bath by 1 - 2°C. It was noticed that uterine horns stored overnight at 4°C rarely showed excessive spontaneous activity.

ii Lack of sensitivity: Although diminished sensitivity was commonly noticed within 2 - 3 hours in many preparations, a few uterine horns did not respond to comparatively large doses of oxytocin from the beginning of the experiment. The sensitivity could be increased in some instances by raising the temperature of the bath, but the resultant responses commonly became of the all or none variety. In the latter part of

the work, preparations which did not respond to 15 m.U of oxytocin at the beginning of the experiment were discarded forthwith.

iii Lack of discrimination: Despite good sensitivity, some preparations responded in all or none fashion, and were thus useless for quantitative work.

In suitable preparations, the sensitivity of the horns to oxytocin varied widely. Most uteri reacted to doses ranging from 1 to 12 m.U. in the 2 ml. bath. The sensitivity thus varied between $\frac{1}{2}$ - 6 m.U./ml.

The precision of the assays can be estimated by the value of λ , (Gaddum, 1953). In the present work, 76 4-point assays were carried out. The value of λ varied between 0.03 and 0.11, the modal value being 0.07. The standard error of the individual assays (λ R) ranged from 5.8 to 15.8.

The accuracy of the method was determined by recovery experiments, in which the unknown (U) was a dilute solution of oxytocin in phosphate buffer prepared by a second observer. The results of 7 such experiments are shown in Table 7, and indicate an average percentage recovery of 102.5 with a mean error of $\pm$ 11.8%.

(2) Oxytocin Recoveries in Zero Time Samples

When zero time samples were assayed against a standard of oxytocin, the percentage recovery varied between 80.6 and 115.4 from the theoretical value of

TABLE 7

No. of Exp.	λ	Estimated U/S %	True U/S %	% Recovery
1	0.08	116.2	125.0	93.0
2	0.08	101.4	100.0	101.4
3	0.10	97.8	83.3	117.4
4	0.05	100.0	108.0	92.6
5	0.07	112.4	130.0	86.5
6	0.05	76.8	66.7	115.0
7	0.09	111.9	100.0	111.9
			Average	102.5
			S.D.	($\pm$ 11.8)

Recovery Experiments on "Unknown" Solutions of Oxytocin

1000 m.U./ml. with an average of 92.4 (± 10.6). There is thus no clear evidence that the presence of deproteinised plasma, placental extract or any of the inhibitors introduces an additional element of error in the results by an effect on either oxytocin or the assay preparation.

(3) Residual Oxytocin in the Control Incubations

Following 60 minutes incubation under the present experimental conditions, the residual oxytocin varied between 9 and 34% in 14 experiments with plasma samples from different subjects, and between 2.5 and 16% in 6 experiments with different placental extracts. The enzyme activity in the two sources were thus approximately comparable.

(4) Effects of Inhibitors

The results of the inhibition experiments with plasma and placental oxytocinase are shown in Table 8. Of the heavy metal ions, inhibition was marked with Zn^{++}, Cu^{++} and Ag^{++} for both placental and plasma enzymes. Parallel results were obtained with Fe^{++} and Sulphydryl inhibitors, where no loss of activity could be detected. Metal-chelating agents however, produced 45.5 - 100% inhibition of plasma oxytocinase, but had no inhibitory effect on the oxytocinase activity of the placental extracts.

CONCLUSIONS

1 Incubation of oxytocin with plasma from pregnant subjects (36-40 weeks gestation) leads to loss of biological activity of the hormone as tested

TABLE 8

Percentage Inhibition (Ranges)

Source of the Enzyme	Cu^{++}	Ag^{+}	Fe^{++}	Zn^{++}	Iodacet-amide	P.Chlor. Mercuric Benzoate	8-Hydroxy quinoline	E.D.T.A.
Placenta	60-91	40-70	0	59-60	0	0	0-14.0	0
No. of Exp.	3	2	2	2	2	2	3	4
Pregnancy Plasma	74.0-100	55.0-92.0	0	46-76	0	0	48-53	46-100
No. of Exp.	3	2	2	3	2	2	2	6

Effect of Inhibitors on Oxytocinase Activities in Pregnancy Plasma and Placental Extracts. All Inhibitors were Employed in a Concentration of 10^{-3}M, except E.D.T.A. (10^{-2}M) All Figures are Approximated to the Nearest Whole Number.

on isolated rat's uterus. The kinetics of the enzymatic reaction follow a first order curve. Inactivation amounts to approximately 30 to 50% of oxytocin in the incubation in the first 10 minutes, and 75 to 90% in 60 minutes.

2 Oxytocin is also inactivated by incubation with extracts of term placentae. The enzymatic activity of 0.4 to 0.5 ml. of a 50% placental extract is approximately equivalent to the activity in one ml. of plasma near to term.

3 The plasma and placental enzymes are stable, and show similar inhibition by heavy metal ions and sulphydryl inhibitors. The plasma factor however, is inhibited by metal-chelating agents which do not affect the activity of the placental factor.

E X P E R I M E N T A L

P A R T

C H A P T E R I I I

A PAPER CHROMATOGRAPHIC STUDY OF THE MECHANISM OF ENZYMATIC DEGRADATION OF OXYTOCIN AND VASOPRESSIN BY PREGNANCY SERA

Tuppy and Nesvadba (1957) employed paper chromatography to study the enzymatic degradation of oxytocin by pregnancy sera. They reported that, following enzymatic inactivation of oxytocin and oxidation of the degradation product, a substantial amount of cysteic acid could be detected by paper chromatography. Tuppy and Nesvadba (1957) thus concluded that pregnancy serum oxytocinase inactivates oxytocin by splitting the peptide bond between the cystine and tyrosine residues. The report, however, lacks all details about the substrate employed, the conditions for incubation, and the paper chromatographic techniques used.

Nevertheless, paper chromatography was chosen for the present study as it helps to separate and identify compounds in µgm amounts by a comparatively simple process. The exact mechanisms involved in this separation are not fully understood, but as pointed out by Smith (1960) they include:

i Continuous partition between the solvent flowing along a filter paper and the water held in it (filter paper contains approximately 15% of its weight of water).

ii Active adsorption of the compounds to the paper which thus acts in a similar manner to alumina columns.

iii Ionic binding and exchange as filter paper has a strong affinity for polar molecules which are held by hydrogen bonding, and can also function as an ion-exchange material due to its content of hydroxyl groups.

The interaction of these various factors results in migration of compounds applied at the origin to different characteristic distances. For convenience, the final position of a substance on the chromatogram is specified by its "R_f" which is the relative migration rate obtained by dividing the distance to which the material has moved from the original point of application by the distance the solvent front has travelled from that point. The relative migration rate (R_f) for a specific compound in a particular solvent is affected by many factors, including the size of the chromatography tank, the atmospheric temperature, type of paper employed, the direction of flow of solvent (ascending or descending), the length of the solvent flow, as well as the pH, and the inorganic salt concentration in the material applied at the origin. The R_f value for a compound is thus not an absolute index (e.g. like the melting point), but is quite reproducible in day to day experiments in the same tank and usually reasonably comparable in different laboratories. Ordinarily, however, pure reference substances are developed under the same conditions for comparison and identification of unknown constituents in a mixture.

The first observations on paper chromatography of posterior pituitary peptides were made by Mueller et al (1951), who employed this technique to investigate the effect of oxidation of purified natural oxytocin by performic acid. The amount of material applied at the origin for chromatography can be calculated as approximately 0.25 mg., and the oxidation product was located with Ninhydrin reagent. Paper chromatography has subsequently been employed to separate and identify the various polypeptide hormones of the neurohypophysis by many workers including Benfey (1953); Schally et al (1958); Acher et al (1958); Heller and Lederis (1958); Boissonnas and Huguenin (1960); Hope et al (1962) and Aroskar et al (1964). Table 9 summarises some of their relevant methods and results when the polypeptides were developed in a solvent of n-Butanol-acetic acid-water. As expected, the results are fairly comparable but not identical.

Preliminary Observations and Design of the Present Experiment.

a Choice of the Solvents

Review of the literature revealed that n-Butanol-acetic acid mixture "Bu-A" (Partridge, 1948) was the most widely employed solvent for characterisation of posterior pituitary polypeptides. The available data could thus provide a basis for comparison. This solvent was thus adopted for the present study. However, the sulphur amino

TABLE 9

Peptide	R_f value	Location agent	Amount employed µgm	Technique	Reference
Oxytocin	0.35-0.40	Ninhydrin	1000	ascending	Benfey, 1953
	0.37	Ninhydrin	unspecified	unspecified	Schally et al, 1958
	0.65	Bromphenol Blue-Mercuric Reagent	unspecified	descending	Acher et al, 1958
	0.59	Platinum Iodide	unspecified	descending	Hope et al, 1962
	0.33	Unspecified	unspecified	descending	Aroskar et al, 1964
	0.37-0.4	Chlorination	5	ascending	Heller and Lederis, 1958
Arginine Vasopressin	0.15-0.18	Ninhydrin	1000	ascending	Benfey, 1953
	0.18	Ninhydrin	unspecified	descending	Schally et al, 1958
	0.19	Chlorination	1 - 6	ascending	Heller and Lederis, 1958
Lysine Vasopressin	0.11	Ninhydrin Folin Chlorination	unspecified	ascending	Boissonnas and Huguenin, 1960
	0.26	Chlorination		ascending	Heller and Lederis, 1958

METHODS AND RESULTS OF PAPER CHROMATOGRAPHY OF POSTERIOR PITUITARY HORMONES IN n-BUTANOL-ACETIC ACID SOLVENT

acids (L-Cysteine, L-Cystine, L-Cysteic acid) which form a basic residue in oxytocin and vasopressin molecules do not run in this solvent and have R_f values very near to zero (Smith, 1960). It was thus decided to use another solvent, Methanol-Pyridine (Smith, 1960) in parallel to enable their identification.

b Choice of Technique for Chromatography

Both ascending and descending techniques have been employed by different workers. However, ascending chromatography techniques required a long time for development, e.g. 16 - 18 hours in Bu-A reported by Heller and Lederis (1958). To cut down experimental time it was decided to employ descending chromatography.

c Choice of Paper

Initial trials were done in both solvents on Whatman No. 1 and Whatman No. 4 chromatography paper. In Bu-A, it was found that a run of 20 cm. required 5 - 6 hours for descending chromatography. The same result was achieved within 2½ - 3 hours on Whatman No. 4 paper with equally good separation. Subsequently all chromatographic experiments in Bu-A were carried out on Whatman No. 4 paper. In parallel experiments with Methanol-Pyridine (Me-P) a 20 cm. solvent run on Whatman No. 1 paper required 2½ - 3 hours. Although Whatman No. 4

paper gave a run of similar length in only 1 - 1½ hours, the resultant separation was less adequate. Subsequently all chromatography work in Me-P was carried out on Whatman No. 1 paper.

d Choice of Location Agent

Table 9 shows the various staining procedures employed to locate the pituitary polypeptides on paper chromatograms, and their reported sensitivity when the data were given in the publication. In the initial experiments, oxytocin solution was applied to a Whatman No. 1 strip as a compact spot in amounts of 2 to 20 µgm (1 - 10 U) and the dry papers were then treated by one of the following location agents:

1 Ninhydrin Reagent

The paper was dipped in Ninhydrin solution in acetone (0.2% wt/v), hung to dry and then heated for 5 minutes at 100°C. A faint pink staining was obtained only with the high doses (20 µgm). The relative insensitivity of Ninhydrin reagent to pituitary polypeptides has been commented upon by Heller and Lederis (1958) who remarked that Ninhydrin only reacts with terminal amino groups. It is thus not the ideal reagent for locating the pituitary cyclo-peptides.

2 Folin's Phenol Reagent

In alkaline medium this reagent gives a violet blue colour in presence of proteins and

peptides containing the phenolic amino acids tyrosine or tryptophan. A solution of synthetic oxytocin gives this reaction. The same principle was introduced by Arimura and Dingman (1959) as a sensitive method for detection of oxytocin and vasopressin on paper chromatograms, using specially treated glass fibre paper. They could stain amounts of 0.5 µgm of the peptides (approximately 0.25 U.). In our experience, 2 µgm of oxytocin could easily be stained on Whatman paper by the Arimura and Dingman method (1959). However, when chromatography was attempted in Bu-A it was very difficult to locate the peptide spot by Folin's reagent spraying even with amounts up to 8 µgms due to heavy bluish background staining of the paper.

3 Chlorination Method

Ryden and Smith (1952) reported that micrograms of peptides (including cyclopeptides) can be detected by chlorination followed by spraying with starch-potassium iodine solution. Reindel and Hoppe (1954) improved this procedure in two ways. First, before exposure to chlorine, they moistened the paper with a mixture of aqueous ethanol and acetone, which prevents background staining. Secondly, they treated the N-Chloro-peptides with a mixture of potassium iodide and

either O-Tolidine or Benzidine in acetic acid and thereby increased the sensitivity of the method. Heller and Lederis (1958) introduced this technique for paper chromatography of oxytocin, vasopressin and their analogues. In the present study, this method proved very satisfactory. Oxytocin (4 µgm) and vasopressin (2 µgm) can be confidently located by this method on paper chromatograms. Heller and Lederis reported an even higher sensitivity (3 µgm for oxytocin and 0.7 µgm for vasopressin).

The reagents required for this reaction were:

Acetone:Absolute Alcohol mixture 1:1 by volume.

Potassium Permanganate solution M/10 (3.2 gm/litre).

HCl 10% (¼ dilution of conc. HCl).

O-Tolidine (reagent grade) in 2% acetic acid (saturated solution).

Potassium Iodide aqueous solution (16.6 gm/litre).

Acetic Acid 2% in water (v/v).

Location of the peptides involved the following steps:

a The dry chromatography strip was wetted in acetone: absolute alcohol mixture and immediately blotted between two sheets of clean filter paper to remove the excess of the reagent.

b The moist strip was then suspended in a plastic

container measuring 22.5 x 22.5 x 9 cm. Twenty mls. of potassium permanganate solution were poured into the bottom of the container, followed by an equal volume of HCl. The lid was replaced and the container rocked gently for 5 minutes (by the stop watch) to ensure adequate mixing of the permanganate and acid. This step was best carried out in the fume cupboard to minimise the spread of chlorine when the lid was removed. In addition to being an irritant, the gaseous chlorine was likely to interfere with the next step.

c At the end of 5 minutes, the chromatography strip was immediately transferred into a staining tray containing equal volumes of O-Tolidine and potassium iodide solutions, freshly mixed together. All parts of the paper strip should be submerged at once for 2 minutes. Peptides appeared as yellow spots quickly changing into violet blue. The staining tray should be kept at some distance from the source of chlorine, otherwise flakes of blue stain form on the surface of the O-Tolidine potassium iodide mixture and smudge the chromatography strip, giving rise to artefacts and making identification of the true spots very difficult.

d The chromatography strip was washed in 2 changes of 2% acetic acid, blotted between two sheets of filter paper and hung up to dry at room temperature.

The colour faded out in daylight within a few hours, and the spots were thus outlined immediately after staining.

In individual experiments, the density of the colour was roughly proportional to the peptide load.

The use of a sensitive location agent in the present study was essential for the following reasons:

1 To minimise the amounts of peptides employed as we only had a very limited supply of the pure highly concentrated oxytocin and vasopressin.

2 Although concentration of the peptides and their cleavage products, by evaporation under reduced pressure, before chromatography was technically possible, this procedure is attended by a risk of polymerisation in case of the posterior pituitary peptides (Ressler, 1958). It was thus decided to avoid this step and work with more dilute mixtures and a sensitive staining agent.

e Observations on the Deproteinisation of Pregnancy Sera.

The high sensitivity of the chlorination method for staining introduced a source of error in our proposed experiment. Pregnancy sera contain 5 - 6 gm % of proteins which are stainable by the Reindel and Hoppe method. Complete and thorough deproteinisation was thus mandatory before the incubated peptides (or their degradation products) could be identified on the paper chromatogram. Experiments were thus carried out to test the efficiency of the various methods for deproteinisation of pregnancy sera. A mixture of equal volumes of pregnancy sera and

phosphate buffer (o.2 M, pH 8) was deproteinised with the following methods:

i Heat coagulation for 5 minutes in a boiling water bath.

ii Acetone precipitation 1:1 by volume.

iii Precipitation with 1:1 volume of 10% and 20% trichloracetic acid.

After shaking the mixtures were centrifuged at 2000 r.p.m. for 10 minutes, and the clear supernatant was tested by paper chromatography, employing the Reindel and Hoppe method for staining. When a load of 200 µl was tested, subsequent chromatography revealed in all instances the presence of several (3 to 5) chlorination positive spots (Fig. 12). A control estimation of the deproteinised mixtures by Folin's reagent (Miller, 1958) also gave substantial reading for these serum "blanks". It thus appeared that simple deproteinisation by any of the above methods was not sufficient for our study. Following incubation with oxytocin and vasopressin it would have been almost impossible to identify with confidence the formation of new compounds on the chromatogram.

Although no attempt was made to identify these chlorination and Folin positive compounds individually, it was suspected that they might be free amino acids of low molecular weight. We therefore tried the effect of dialysis. Pregnancy sera were dialysed in Visking cellulose seamless tubing against distilled water for 3

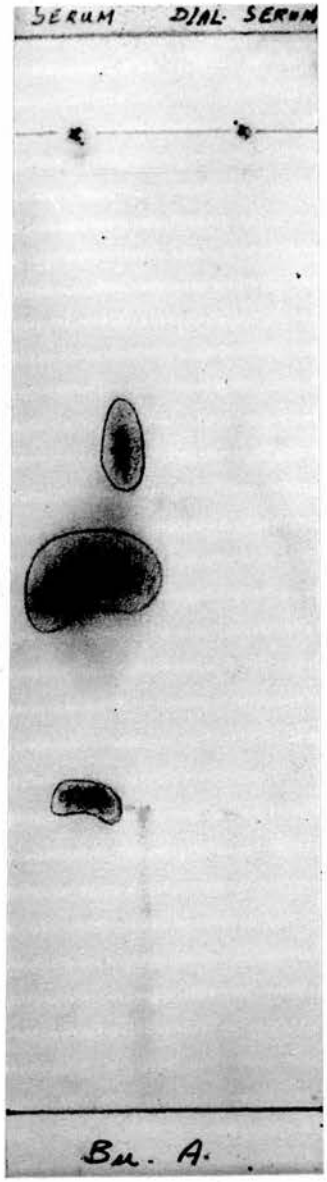


Figure 12 Paper Chromatogram Showing Effect of Preliminary Dialysis of Sera. Load 200 µL. of Trichloracetic Acid De-Proteinised Serum. Descending Chromatography in Bu-A.

hours at 4°C (in the refrigerator). The sera were then diluted with phosphate buffer (1:1) and deproteinised with 20% trichloracetic acid, equal and half volumes. Following centrifugation, the clear supernatant was chromatographed employing a load of 200 µl. Control runs of the same serum blanks without dialysis were also done.

After development in Bu-A the papers were dried and stained by the Reindel and Hoppe method. With few exceptions, the dialysed serum blanks showed no stainable chlorination positive compounds, in contrast to the un-dialysed controls (Fig. 12). Dialysis was thus instituted as a basic step in subsequent experiments. Prior to incubation with oxytocin or vasopressin all sera were dialysed. A serum "blank" was prepared (50% in phosphate buffer) deproteinised by half volume of trichloracetic acid, and tested by paper chromatography. Only sera which produced a clean chromatogram devoid of interfering compounds were accepted for subsequent incubation.

f Effect of Dialysis on Oxytocinase Activity.

According to Werle et al (1941) and Page (1946) the oxytocinase activity of pregnancy sera (assayed biologically) is not affected by dialysis against water. Table 10 summarises the results on 10 pregnancy sera. The oxytocinase activity was estimated by the chemical method described by Tuppy and Nesvadba (1957) before and after dialysis for 3 hours against distilled water at 4°C. A slight loss of activity of less than 5% was noted in all

TABLE 10

Sample No.	mg. β-naph/100 ml. serum/hour Before Dialysis	After Dialysis	% Recovery
1	9.2	8.9	96.7
2	7.5	7.3	96.0
3	6.2	5.9	95.0
4	5.6	5.4	96.4
5	5.7	5.6	98.3
6	10.9	10.5	96.3
7	6.9	6.6	95.7
8	8.3	7.9	95.2
9	4.7	4.5	95.8
10	8.8	8.4	95.5
		Average	96.1
		S.D.	(±1.0)

Effect of Dialysis on Serum Cystine Aminopeptidase Activity "Oxytocinase"

The Results are Expressed as mg. β naphthylamine/ 100 ml. Serum/Hour. (See page 100)

instances which could be accounted for by the inevitable slight volume increase and dilution of sera during dialysis.

g R_f Values of Oxytocin and Vasopressin.

The chromatographic behaviour of oxytocin and vasopressin(s) was studied in two solvents.

i n-Butanol-Water-Acetic Acid 40:10:50 (on Whatman No. 4 paper)

ii Methanol-Water-Pyridine 160:40:8 (on Whatman No. 1 paper)

The peptides employed were synthetic Oxytocin, synthetic Lysine-vasopressin, synthetic Arginine-vasopressin and natural Lysine-vasopressin (Pitressin).

Amounts of 2 - 4 units of the peptides (4 - 8 µgms approx.) were applied at the origin and the chromatograms were developed to a length of 20 cms. by the descending technique in $2\frac{1}{2}$ - 3 hours. The peptides were located by the Reindel and Hoppe chlorination method.

The results are shown in Figs. 13 and 14 and Tables 11 and 12. Single spots were obtained with all pure synthetic compounds in both solvent media. Commercial Pitressin solutions gave a single spot in Bu-A which corresponded well with that of synthetic Lysine-vasopressin. In Me-P, however, it showed as 2 - 3 spots, one of which had the same R_f as the pure synthetic vasopressins. The Pitressin solution was thus regarded as unsuitable for the present chromatographic study , due to its high content of impurities.

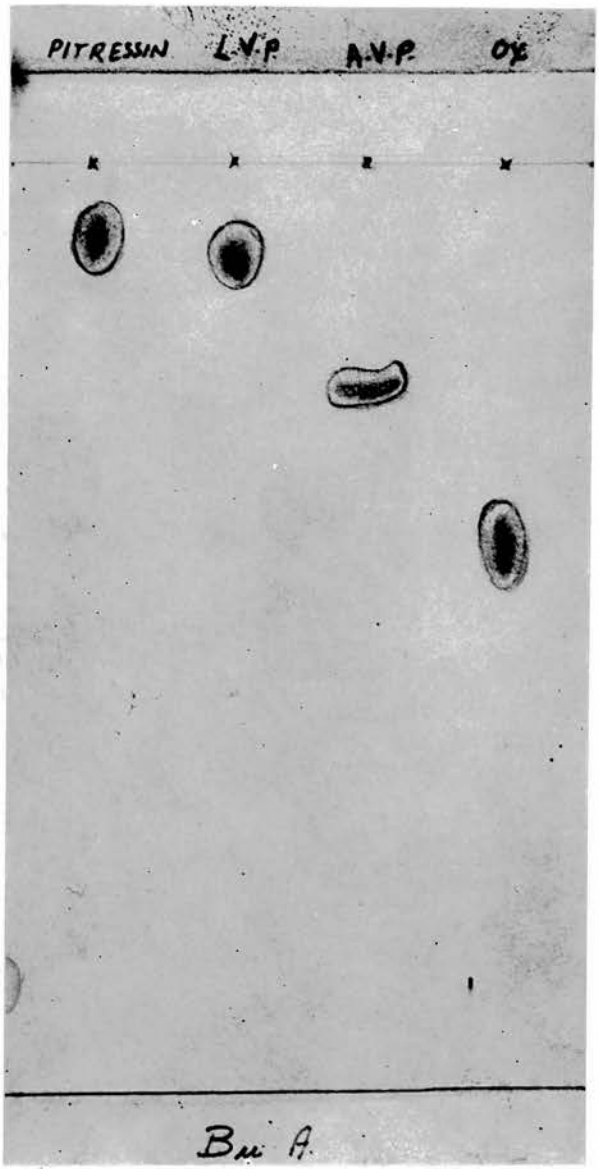


Figure 13 Chromatography of Posterior Pituitary Hormones in Bu-A. Loads: Synthetic Oxytocin (Ox), 4 U; Synthetic 8-Arginine-Vasopressin (A.V.P.) 2 U; Synthetic 8-Lysine Vasopressin, 2 U; Pitressin, 2 U.

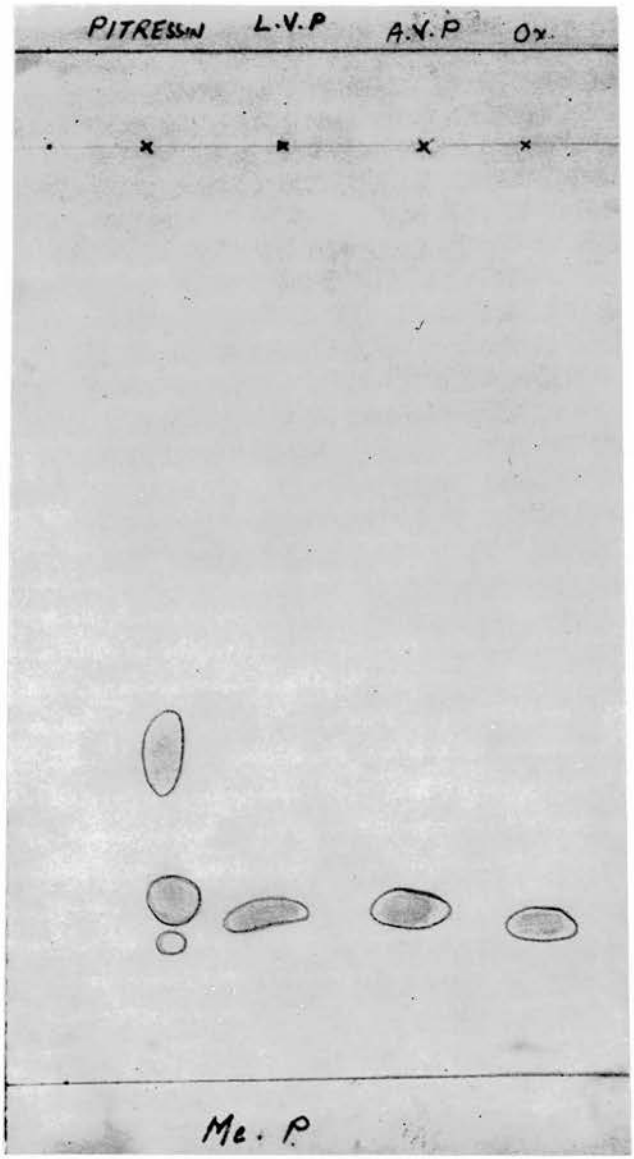


Figure 14 Chromatography of Posterior Pituitary Hormones in MeP. Same Loads as Figure 13.

TABLE 11

Peptide	No. of Exps	R_f Average	Range
Oxytocin	14	0.41	0.37-0.43
Lys.vasopressin	5	0.12	0.10-0.15
Arg.vasopressin	2	0.23	0.22-0.24

R_f Values of Synthetic Oxytocin and Vasopressins in a Solvent of n-Butanol-H_2O-Acetic acid (40:10:50)

TABLE 12

Peptide	No. of Exps.	R_f	
		Average	Range
Oxytocin	8	0.82	0.78-0.85
Lys. vasopressin	5	0.82	0.77-0.87
Arg. vasopressin	2	0.81	0.80-0.82

R_f Values of Synthetic Oxytocin and Vasopressins in a Solvent of Methanol-H_2O-Pyridine (160:40:8)

The peptides have different R_f values in Bu-A, but migrate to the same distance in Me-P. The results of chromatography in Bu-A are in good agreement with those obtained by other workers, as shown in Table 10.

h Effect of Oxidation on Oxytocin and Lysine-Vasopressin

Controlled oxidation of oxytocin and vasopressin by performic acid results in disruption of the disulphide bridge between the two half-cystine residues (in positions 1 and 6) with formation of an open chain compound which gives a single spot on paper chromatography. No free cysteic acid is liberated from the molecule (Mueller et al, 1951).

A simpler method for oxidation was tried by employing 30% Hydrogen Peroxide solution as described by Smith (1960). Fresh peroxide solution (0.1 ml.) was added to 0.4 ml. of synthetic oxytocin (200 U/ml., pH 3.5). All solutions were precooled to 4°C, allowed to stand after mixing in an ice-water bath for 10 minutes, and then immediately applied to Whatman filter paper strips for chromatography. L-cysteic acid (8 µgm) was also run on the same strips as a marker. The results are shown in Figs. 15 and 16. A single oxidation product was obtained in all cases. The oxidation products of both oxytocin and lysine vasopressin had lower R_f values in both Bu-A and Me-P than the parent molecules. No free L-cysteic acid was identified in the oxidation mixtures.

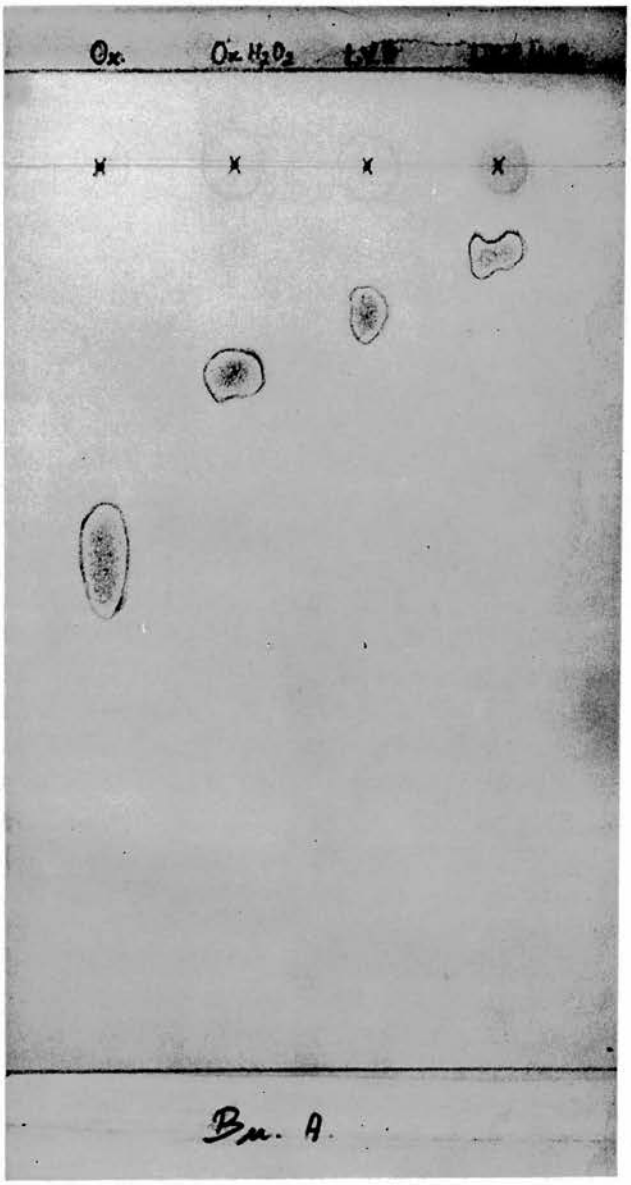


Figure 15 Effect of H_2O_2 Oxidation of Synthetic Oxytocin (Ox., Ox. H_2O_2), and Synthetic 8-Lysine-Vasopressin (L.V.P., L.V.P. H_2O_2). Loads 4 U. (approx.). Descending chromatography in Bu-A.

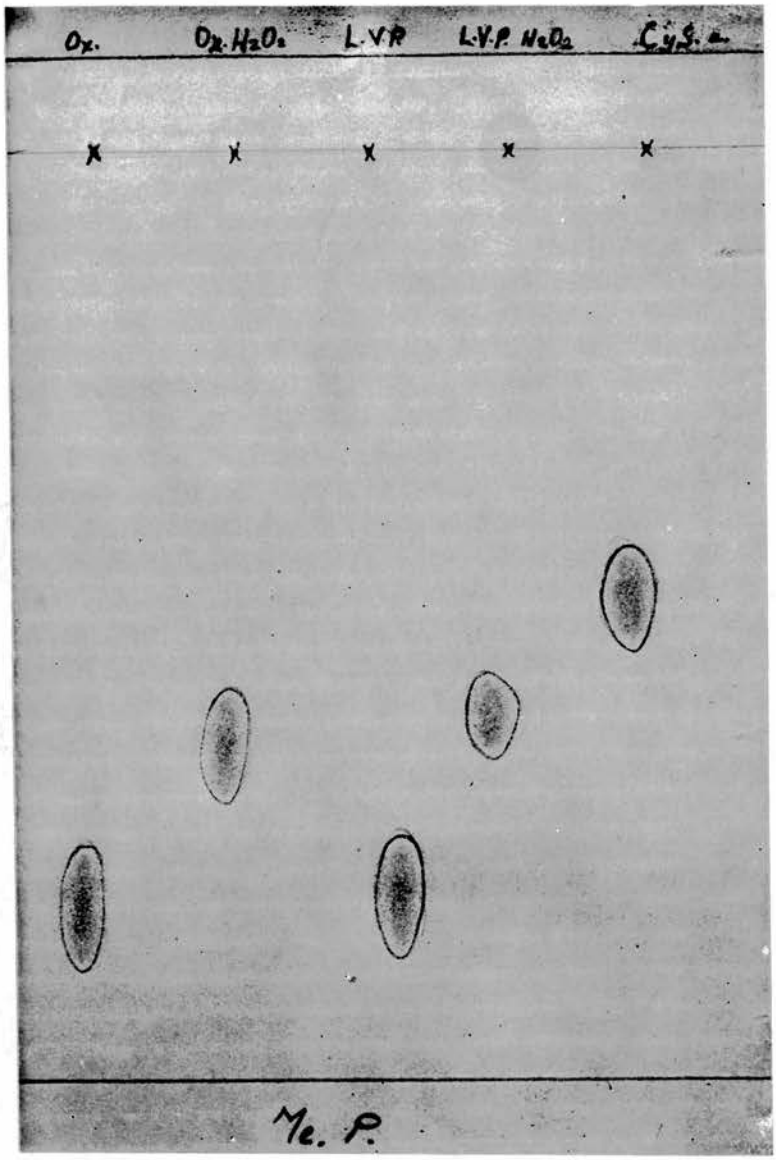


Figure 16 Effect of H_2O_2 Oxidation of Synthetic Oxytocin (Ox., Ox. H_2O_2) and Synthetic 8-Lysine-Vasopressin (L.V.P., L.V.P. H_2O_2). Loads 4 U. (approx.) L-Cysteic Acid (Cys) Run as a Marker in a Load of 8 µgm. Descending chromatography in Me-P.

1 The Final Experimental Design.

Having established the suitable conditions for chromatography of oxytocin, vasopressin and their oxidation products, and for satisfactory deproteinisation of pregnancy sera, the enzymatic effects of pregnancy sera on either hormone were studied according to the following design:

i Selection of sera of high oxytocinase activity.

ii Incubation with oxytocin or lysine vasopressin at pH 7.8.

iii Deproteinisation of the incubation mixture.

iv Oxidation of part of the protein free supernatant.

v Separation and identification of the enzymatic degradation product(s) and their oxidation yield by paper chromatography.

Material and Methods

1 Hormonal Substrates

The synthetic chemically pure polypeptide hormones employed in this study were obtained in the following forms.

i Synthetic Oxytocin: concentrated solutions were obtained from Parke Davis and Co. (198.95 U/ml., Batch No. 45985, 10 ml. vial) and Sandoz Ltd., (450 U/ml., one ml. ampoules). The solvent was a sodium acetate-acetic acid buffer pH 3.5 (approx.) in both preparations.

ii Synthetic Lysine Vasopressin: concentrated solution containing 120 U/ml. watery solution acidified by drops of dil. HCl to pH 3.5. This preparation was obtained from Professor J. Rudinger, Prague, in one ml. ampoules.

2 Buffer

Sodium phosphate buffer pH 8, ionic strength o.2 M. The relatively high ionic strength was required to neutralise the acidity of the hormonal substrate solutions.

3 Chromatographic Solvents

n-Butanol-Water-Acetic acid 40:10:50 (Partridge, 1948). The mixture was thoroughly shaken and allowed to stand overnight. The upper solvent layer was used for chromatography.

Methanol-Water-Pyridine 160:40:8 (Smith, 1960). The mixture formed a monophasic solvent. Solvent stock solutions were prepared every 3 - 4 weeks and stored in the refrigerator. The solvent mixtures in the chromatography trough were changed weekly.

4 Other Chemicals

Hydrogen Peroxide 30% (wt/v)

Trichloracetic Acid 20% in water

Chemicals for the Reindel and Hoppe location method (page 74)

Chemicals for chemical estimation of oxytocinase (page 97)

5 Chromatography Apparatus

Two Shandon glass chromatography tanks were employed

throughout. The larger tank measured 50 x 50x 20 cm. and was used for descending chromatography in Me-P. A smaller tank, 30 x 54 x 18 cm. was employed with Bu-A. Initially and with every change of solvent, the lid was replaced and the tank was left to saturate and equilibrate overnight.

Chromatography strips were cut out from larger sheets of Whatman No. 1 and No. 4 filter paper for chromatography. Strips employed were 34 cm. long and of suitable width so as to allow application of the various solutions for chromatography at 3 cm. distances on the origin line, leaving a free margin of 2 cm. at the sides. The papers were marked at 20 cm. from the origin line to indicate the end of the run.

Micropipettes (10 µl) were used to apply the material for chromatography at the point of origin so as to ensure a compact small spot. A current of warm air from a hair drier was employed to help dry the paper between the applications.

Experimental Procedure

1 Collection of Blood Samples

Venous blood samples (15 ml.) were obtained from patients 36 or more weeks pregnant. Samples were obtained whenever possible from patients with twin pregnancy to ensure a high oxytocinase activity of sera (Page et al, 1961).

2 Separation and Dialysis of Sera

After clotting of blood, sera were separated by

spinning at 2000 r.p.m. for 10 minutes. The supernatant serum was decanted. A suitable volume (4 - 6 ml.) was poured into a prewetted Visking cellulose dialysis tubing. Dialysis was carried out in a 5 l container against distilled water for 3 hours at 4°C.

3 Selection of Sera for Incubation

Subsequent incubations with hormonal substrates was limited to serum samples which fulfilled the following two conditions:

i A high oxytocinase activity as tested by Tuppy and Nesvadba's chemical method (1957).

ii A chromatographically "clean" blank as tested in the following way: 1 ml. of dialysis serum was mixed with 1 ml. phosphate buffer and 1 ml. trichloracetic acid. Following mixing and centrifugation, the supernatant was decanted and applied to Whatman No. 4 paper strips in loads of 200 µl. The strips were developed in Bu-A and stained by chlorination. Sera which gave no stainable ingredients following this treatment were considered reliable for subsequent incubation.

4 Incubation with Oxytocin and Vasopressin

Incubations were carried out under the following conditions:

1.5 ml. dialysed serum + 0.75 ml. phosphate buffer + 0.75 ml. of either oxytocin (200 U/ml.) or lysine vasopressin (120 U/ml.). A drop of chloroform was added as preservative and the reaction of the mixture was tested

by B.D.H. pH paper. In all cases it was found to lie between 7 - 8.

The test mixture (T) was incubated at a temperature of 37°C (± 0.5) for 24 hours.

A control (C) was prepared in all experiments with the same composition, and was stored for a similar length of time at 4°C.

5 Deproteinisation

At the end of 24 hours, the test and control mixtures we deproteinised by ½ volume of 20% trichloracetic acid. The tubes were shaken and then centrifuged (2000 r.p.m. for 10 mins). The clear supernatant was decanted into a clean test tube and stored at 4°C.

6 Oxidation

This was carried out immediately before chromatography. A volume of 0.8 ml. of the deproteinised incubation mixture (T) was treated by 0.2 ml. of hydrogen peroxide 30% in a micro-test tube. The tube was shaken and then stood in the ice-water bath for 10 minutes. The mixture (TH_2O_2) was then applied to the paper strips for chromatography.

7 Chromatography

The following solutions were run together on the same strip:

Control (C), Incubation Mixture (T), oxidised Incubation Mixture (TH_2O_2), L-Cysteic acid "Cys" (8 µgm) and TH_2O_2 + Cys. on the same origin point.

The loads employed were 200 µl. in the oxytocin

incubations and 300 µl. in the vasopressin experiments.

Two strips were employed for each experiment, one for development in Bu-A (Whatman No. 4) and the other for development in Me-P (Whatman No. 1).

A twenty cm. run in either solvent was obtained within $2\frac{1}{2}$ - 3 hours. The papers were then hung to dry and stained by the Reindel and Hoppe method.

Results

1 Effect of the Experimental Procedure on the Chromatographic Behaviour of Oxytocin and Vasopressin

Table 13 compares the R_f values of oxytocin and lysine vasopressin applied directly to the chromatography paper as pure compounds with the values obtained from the control mixtures (C). No significant differences are seen when Me-P is used for developing the chromatogram. In Bu-A, oxytocin also has the same R_f in contrast to lysine vasopressin which had a significantly smaller R_f value in the controls as compared to the pure substrate. The effect may be due to the high acidity of the deproteinised control mixtures ($_pH < 1$) resulting from the use of trichloracetic acid for deproteinisation. The effect is shown in Fig. 17 when lysine vasopressin (4 U.) is run as a pure substrate and in a control mixture in Bu-A.

2 Effect of Incubation with Pregnancy Sera

Following incubation with pregnancy sera, chromatography of the deproteinised test mixture (T) revealed in all instances substantial degradation of oxytocin and lysine vasopressin as evidenced by fading of their

Figure 17 Descending Chromatography of Lysine-Vasopressin as Pure Compound (A.V.P., 3 U.), and in a Control Mixture (C., 300 µL.). Solvent: Me-P.

TABLE 13

Peptide	R_f in Bu-A	R_f in Me-P
Oxytocin	0.41	0.82
Oxytocin in Control Mix.	0.43	0.83
Lysine-Vasopressin	0.12	0.82
Lysine-Vasopressin in Control Mix.	0.09	0.79

R_f Values of Oxytocin and Lysine-Vasopressin Run as Pure Compounds and in the Deproteinised "Control" Mixtures

chromatographic representative spot (as compared with the control C). In most cases, however, it was still possible to identify traces of the hormones in the chromatogram.

A new product was shown on all chromatograms which had a higher R_f value than the parent hormone in Bu-A, and smaller value in Me-P (Figs. 18 and 19). Tables 14 and 15 summarise the R_f values of the main cleavage products following incubation of oxytocin and vasopressin with pregnancy sera.

With oxytocin incubations a faint second new spot was obtained occasionally when the test mixture was run in Bu-A with an R_f value very close to zero.

3 Effect of Oxidation on the Enzymatic Cleavage Products of Oxytocin and Vasopressin

On chromatography of the oxidised test mixture (TH_2O_2) of oxytocin in Bu-A solvent, the enzymatic cleavage product (R_f:0.81) disappeared and a single spot was seen with an R_f very close to zero, (0.032). The results are shown in Fig. 18_a and Table 14. It must be noted that in this solvent L-cysteic acid also had a very low R_f (0.03) as shown in the same figure. With Me-P, however, chromatography of the oxidised test mixture of oxytocin revealed two components with average R_f value of 0. 5 and 0.61. Fig. 18_b also shows that L-cysteic acid had the same R_f value as the slower moving of the two components. Furthermore, when the oxidation mixture and L-cysteic acid were applied to the same point of origin, the two oxidation components were again seen with the proximal

Figure 18a Paper Chromatography of Synthetic Oxytocin, and its Products on Enzymatic Degradation and Further Oxidation; Load of 200 µL. of Control "C", Test "T", and Oxidised Test "TH_2O_2". L-Cysteic Acid (8 µgm.) Run as a Marker: alone "Cys" and with the Oxidised Test Mixture (TH_2O_2 + Cys.). Chromatogram Developed in Bu-A.

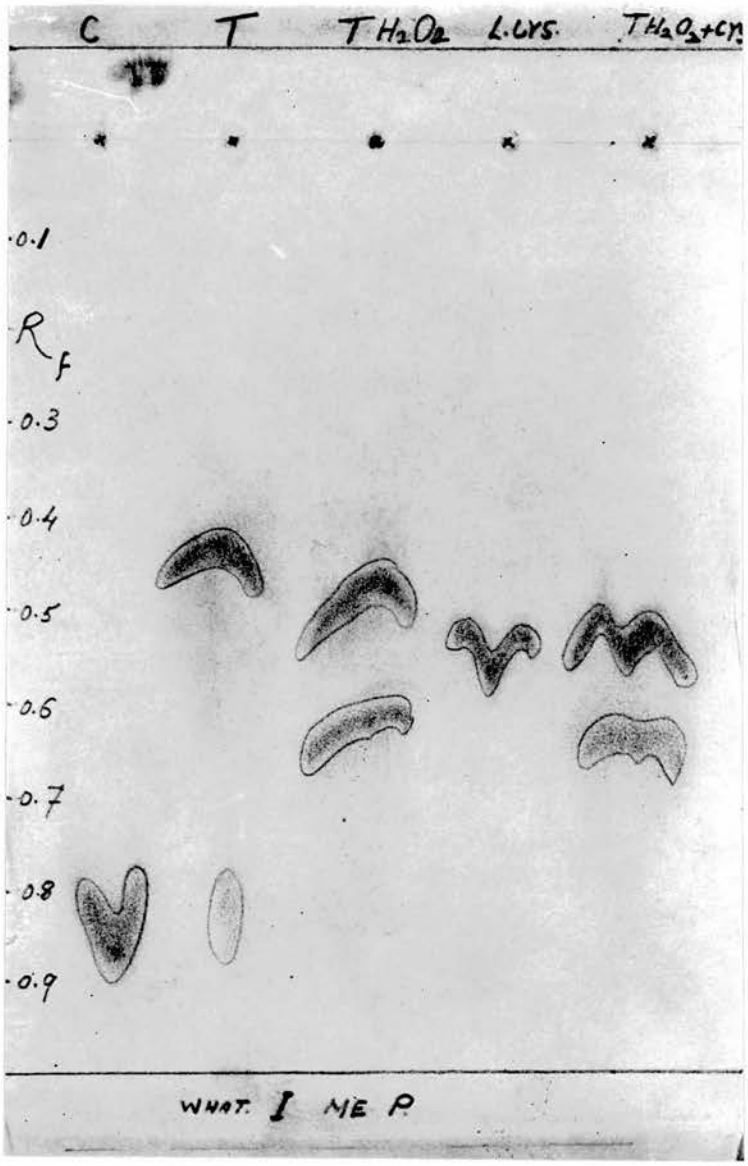


Figure 18b Paper Chromatography of Synthetic Oxytocin, and its Products on Enzymatic Degradation and Further Oxidation; Loads of 200 µL. of Control "C", Test "T", and Oxidised Test "TH_2O_2". L-Cysteic Acid (8µgm.) Run as a Marker: alone "Cys" and with the Oxidised Test Mixture (TH_2O_2 + Cys.). Chromatogram Developed in ^{2}Me-P.

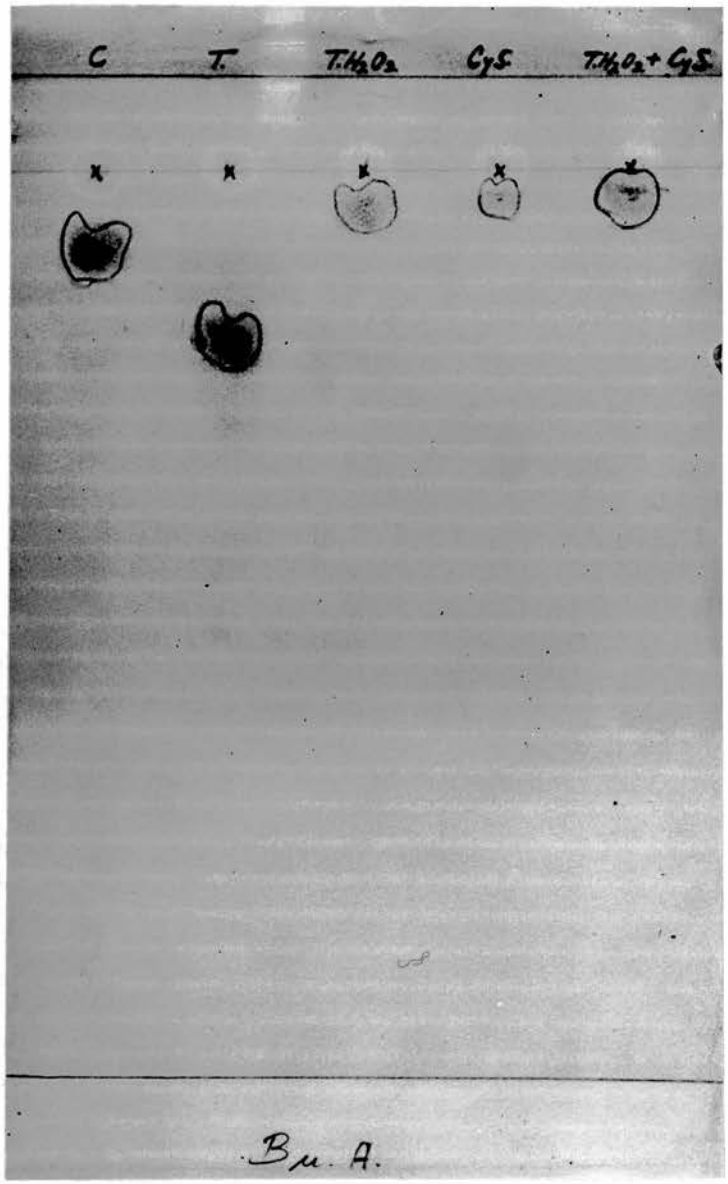


Figure 19a Paper Chromatography of 8-Lysine-Vasopressin and its Products on Enzymatic Degradation and Further Oxidation. Loads of 300 µL. of Control "C", Test Mixture "T", and Oxidised Test Mixture "TH_2O_2". L-Cysteic Acid (8 µgm.) Run as a Marker: alone "Cys" and with the Oxidised Test Mixture (TH_2O_2 + Cys). Chromatogram Developed in Bu-A.

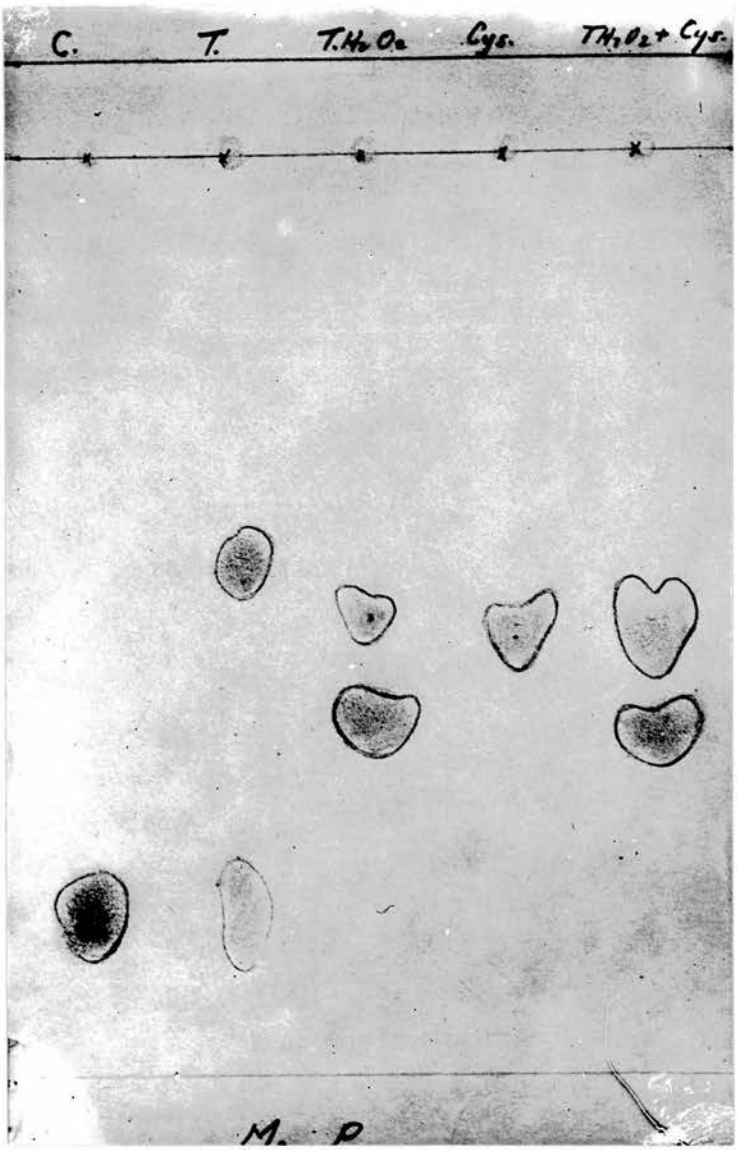


Figure 19b Paper Chromatography of 8-Lysine-Vasopressin and its Products on Enzymatic Degradation and Further Oxidation. Loads of 300 µL. of Control "C", Test Mixture "T", and Oxidised Test Mixture "TH_2O_2". L-Cysteic Acid (8 µgm.) Run as "a" Marker: alone "Cys" and with the Oxidised Test Mixture (TH_2O_2 + Cys.). Chromatogram Developed in Me-P.

TABLE 14

Material	No. of Exp.	Bu-A Solvent R_f	Me-P Solvent R_{f1}	Me-P Solvent R_{f2}
Control "C"	8	0.43 (0.40-0.45)	0.83 (0.80-0.88)	-
Test "T"	8	0.84 (0.75-0.89)	0.44 (0.39-0.48)	-
Oxidised Test "TH_2O_2"	8	0.032 (0.02-0.05)	0.50 (0.43-0.54)	0.61 (0.58-0.65)
L-Cysteic a. "Cys"	8	0.03 (0.03-0.05)	0.50 (0.43-0.54)	-

R_f Values of Oxytocin in Control Mixtures "C" and the Degradation Products after Incubation with Pregnancy Sera "T" and Further Oxidation "TH_2O_2" . Load of 200 µl. L-Cysteic acid "Cys" was Run in all Experiments as a Marker, load 8 µg.

TABLE 15

Material	No. of Exp.	Bu-A Solvent	Me-P Solvent	
		R_f	R_{f1}	R_{f2}
Control "C"	4	0.09 (0.70-0.14)	0.79 (0.78-0.80)	-
Test "T"	4	0.22 (0.175-0.275)	0.44 (0.38-0.49)	-
Oxidised Test "TH_2O_2"	4	0.03 (0.02-0.04)	0.48 (0.46-0.50)	0.59 (0.57-0.61)
L-Cysteic a. "Cys"	4	0.03 (0.02-0.04)	0.48 (0.46-0.50)	-

R_f Values of Lys-Vasopressin in Control Mixtures "C" and the Degradation Products after Incubation with Pregnancy Sera "T" and Further Oxidation "TH_2O_2" Load of 300 µl. L-Cysteic acid "Cys" was Run in all Experiments as a Marker, load 8 µg.

component showing intense staining by the superimposed L-cysteic acid spot.

In similar experiments with lysine vasopressin, a closely parallel pattern was observed. On oxidisation of the test mixture the enzymatic cleavage product which had an average R_f value of 0.22 in Bu-A was replaced by one spot with a low R_f as seen in Fig. 19_a and Table 15. In Me-P solvent, two oxidation components could be recognised, the slower one with the same R_f as L-cysteic acid. In this latter solvent medium the chromatographic behaviour of oxytocin, lysine vasopressin and their cleavage and oxidation products was nearly identical.

Conclusions

The key reaction in these experiments is the oxidation of the polypeptide hormones which is known to disrupt the pentapeptide ring by attacking the disulphide bond between the two half cystine residues (in positions 1 and 6). The result is a single open chain polypeptide with 9 amino acid components including cysteic acid residues in positions 1 and 6. This compound had a different R_f to that of the parent intact hormone as shown in our preliminary experiments. However, oxidation of oxytocin or vasopressin does not lead to the liberation of cysteic acid.

By subjecting oxytocin and vasopressin to enzymatic cleavage by pregnancy sera, the present experiments revealed the formation of one main single compound. On subsequent oxidation it has been shown that free cysteic acid is split off the enzymatic cleavage products of both

oxytocin and lysine vasopressin. As this does not happen with the intact cyclopeptide hormones, it can be concluded that enzymatic degradation by pregnancy sera must have resulted in cleavage of the peptide bond between terminal half cystine (position 1) and the adjoining tyrosine (position 2) residues. The findings are interpreted in Fig. 20. The enzymatic cleavage of oxytocin and vasopressin by pregnancy sera thus appears to be an identical mechanism.

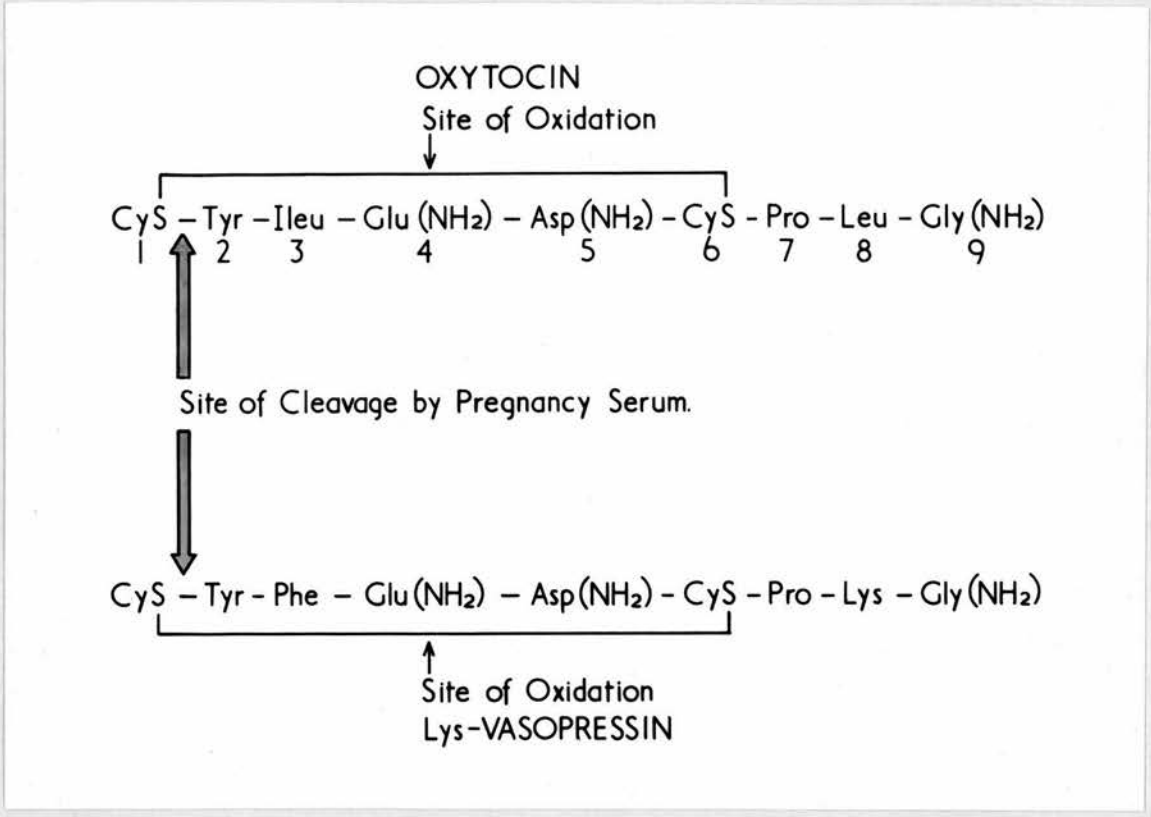


Figure 20 Interpretation of the Chromatographic Findings, Showing the Site of Cleavage of the Oxytocin and 8-Lysine Vasopressin Molecules by the Pregnancy Serum Enzyme, and the Effect of Subsequent Oxidation by H_2O_2.

EXPERIMENTAL PART

CHAPTER IV

CHEMICAL METHODS FOR ESTIMATION OF PREGNANCY SERUM OXYTOCINASE

Tuppy and Nesvadba (1957) introduced the synthetic compound L-Cystine-di-β-naphthylamide as a suitable substrate for the chemical estimation of pregnancy serum oxytocinase. In this compound (Fig. 21) amino-terminal half-cystine residues are linked to β-naphthylamine by peptide bonds, a configuration similar to the N-terminal amino acid sequence of oxytocin, where the enzymatic splitting of the hormone occurs. When incubated with pregnancy sera, the synthetic substrate was split with the liberation of β-naphthylamine. The free β-naphthylamine was estimated colorimetrically by a modified Bratton-Marshall reaction (Bratton and Marshall, 1939). Colour development by this method involved diazotisation followed by coupling with N-(1 Naphthyl) ethylene-diamine-dihydrochloride. The intensity of the violet blue azo dye thus formed was a function of the concentration of β-naphthylamine over a wide range of spectrophotometric readings. The amount of β-naphthylamine released by enzymatic splitting could therefore be determined by comparison to a standard solution of β-naphthylamine. The details of the method for colour development were essentially similar to the procedure originally described by Green et al (1955). The chemical reactions involved are illustrated in Fig. 22.

Tuppy and Nesvadba (1957) also investigated the kinetics of the enzymatic splitting of L-Cystine-di-β-naphthylamide by pregnancy sera, and characterised the optimal conditions for the enzyme assay.

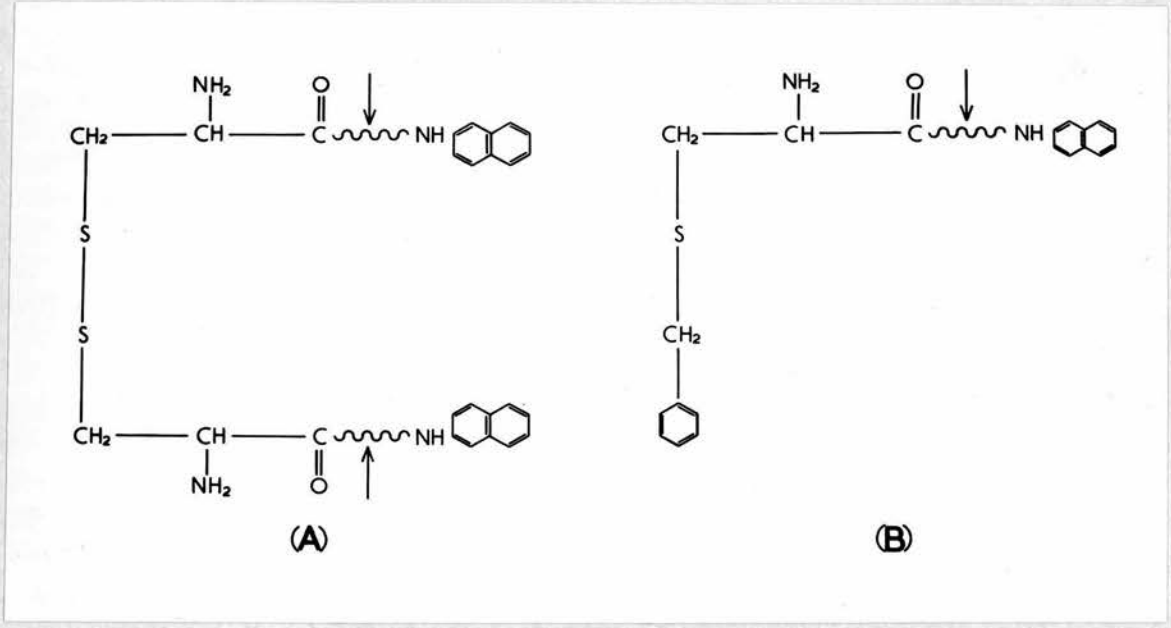
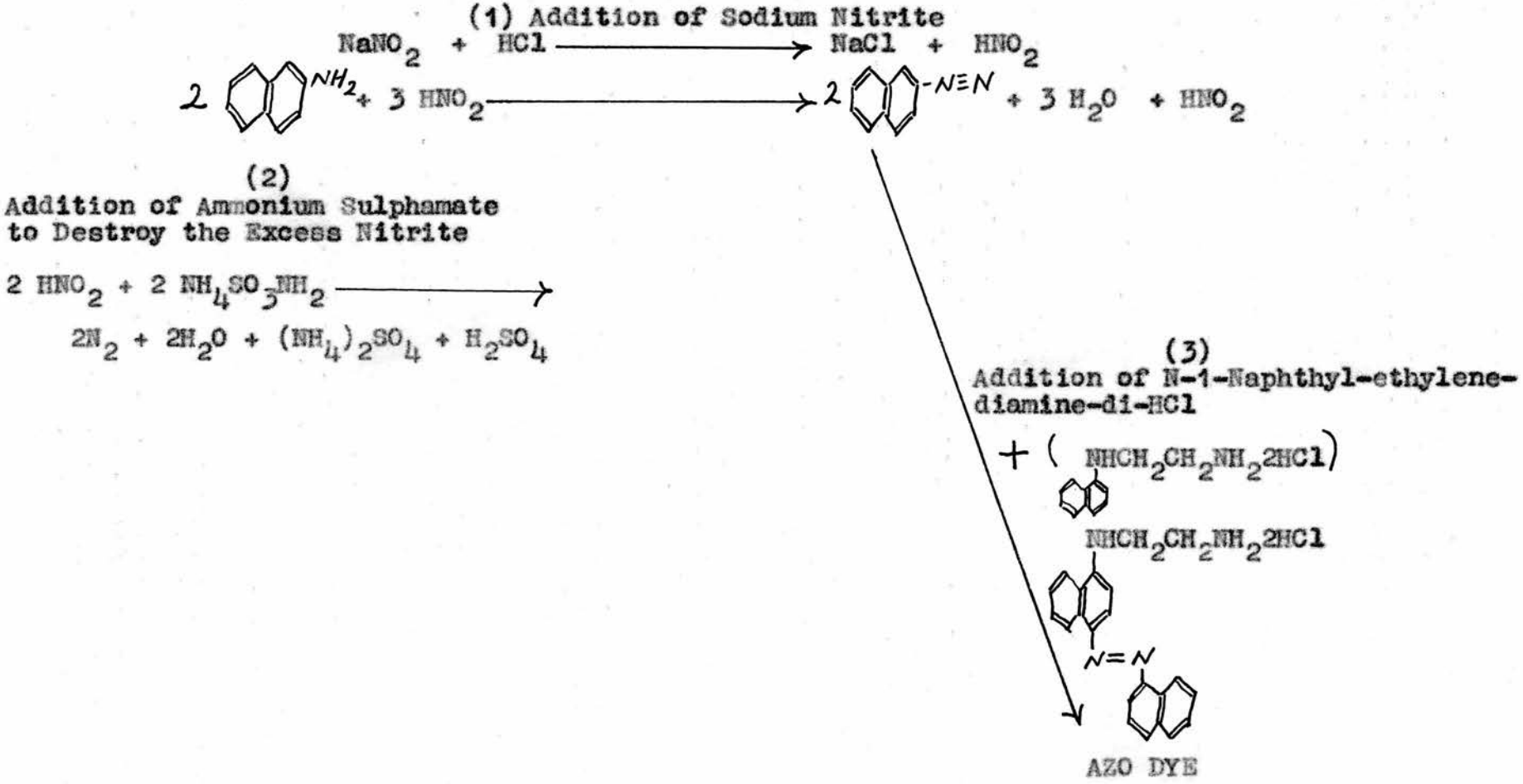


Figure 21 Chemical Formulae of Synthetic Compounds Used for Pregnancy Serum Oxytocinase Estimation. A. L-Cystine-di-β-naphthylamide; B. S-Benzyl-cysteinyl-β-naphthylamide. Sites of enzymatic cleavage indicated by arrows.

Figure 22 Chemical Reactions in Spectrophotometric Estimation of β-naphthylamine (in HCl)



Under these conditions, the kinetics of the reaction followed a zero order form, and the activity was expressed by the number of milligrams of β-naphthylamine liberated by 100 ml. serum per hour. The chemical method was reported to be more simple, accurate and precise than the biological methods for oxytocinase assay.

In 1964, Tuppy and Wintersberger introduced the compound S-Benzyl-Cysteine-β-Naphthylamide (Fig. 2?) as an alternate synthetic substrate for oxytocinase estimation. Following incubation with pregnancy sera, β-naphthylamine was released and was measured fluorimetrically (Tuppy, 1965). This substrate is more soluble in the incubation mixture than the previous one, and the method was reported to be more sensitive and of better specificity.

This part of the work was undertaken to investigate the following problems:

1 To examine the kinetics of the enzymatic splitting of L-Cystine-di-β-naphthylamide by sera of women in late pregnancy.

2 To investigate the validity of the chemical methods for oxytocinase estimation by:

I Studying the splitting of the synthetic substrate L-Cystine-di-β-naphthylamide by sera of pregnant animals and non-pregnant females, in which oxytocinase has been reported absent (Page, 1946; Werle et al, 1950).

II Examination of the effect of inhibitors on the enzymatic splitting of the same substrate by pregnancy sera and comparing the results to the data reported in Chapter II.

III Examination of the distribution of the enzymatic activity towards the synthetic substrate in the various protein fractions of pregnancy sera obtained by ammonium sulphate precipitation and paper electrophoresis, and comparing the results with the published data on oxytocinase (Semm and Werle, 1956).

3 To compare the two available synthetic substrates for oxytocinase estimation.

Material and Methods

Blood samples were collected from animals and human volunteers as described on page 46. Sera were separated by spinning and assayed for enzymatic activity within 48 hours of collection.

(1) Chemical Estimation of Enzymatic Activity on L-cystine-di-β-naphthylamide substrate

The method described by Tuppy and Nesvadba (1957) was employed with minor modifications. The following is an account of the procedure -

Reagents

Substrate Solution: L-cystine-di-β-naphthylamide, 135 mg. was dissolved in 50 ml. of 0.012 N HCl, with

moderate warming. The clear solution was diluted to 100 ml. with distilled water and stored in the refrigerator. The substrate was obtained from Sanabo Company, Anton Scharff Gasse 7, Vienna.

Buffer

Veronal buffer pH 7.9, ionic strength 0.046 M (page 45).

Standard Solution

β-naphthylamine (E. Kodak Co.) 40 mg. was dissolved in 500 ml. 0.012 N HCl with warming and stirring, and diluted to one litre with distilled water. The solution was stored in the dark.

Other Reagents

Trichloracetic acid, 10 per cent (w/v), a mixture of HCl (0.36 N) and acetone (reagent grade) in the ratio 2:1; sodium nitrite, 0.1 per cent aqueous; N-1-naphthyl-ethylenediamine-dihydro-chloride (E. Kodak Co.) 0.1 per cent aqueous.

Fresh solutions of the standard as well as the reagents for colour development were prepared every 2 to 3 weeks.

Method

Serum, 0.6 ml. was diluted with 0.9 ml. water and 3.0 ml. veronal buffer; 0.75 ml. of the diluted serum was pipetted into each of 3 micro test tubes. The first of the three tubes served as the zero time control. Into it was pipetted 0.25 ml. of substrate solution followed immediately by 1.0 ml. of 10 per cent trichloracetic acid. The remaining tubes (duplicates) were placed in a water

bath at 37°C, and 0.25 ml. substrate solution was added to each. After 4 hours at 37°C, the reaction was stopped by the addition of 1 ml. of 10 per cent trichloracetic acid. After shaking, the tubes were centrifuged, and 1.0 ml. of the clear supernatant solution was pipetted into a 20 x 125 mm. test tube. (If time did not permit development of colour on the same day as the enzyme reaction, these tubes were stoppered and stored in the refrigerator overnight).

A reagent blank and standards were set up as follows: 1.5 ml. of water was mixed with 3.0 ml. buffer, and 0.75 ml. of the resulting solution was pipetted into each of three 20 x 125 mm. test tubes. To the reagent blank was added 0.25 ml. of water, and to the standard, 0.25 ml. of the standard -naphthylamine solution.

Colour Development

This was carried out in a semi-darkened room. To each tube containing 1 ml. of supernatant (or standard) was added 9 ml. of the HCl-acetone mixture. The following additions were made at 3 minute intervals (using a stop-watch), and the tubes were mixed by shaking after each addition: 1 ml. sodium nitrite, then 1 ml. ammonium sulfamate, and finally 1 ml. N-1-naphthylehylene-diamine-dihydro-chloride. The tubes were stoppered and set in the incubator in the dark at 37°C for 4 hours. The maximal colour was reached after 3 - 4 hours and remained constant for several hours. After cooling to room temperature, the samples were read in a spectrophotometer (Unicam S.P. 600) at 565 mμ.

Calculation

The enzymatic activity of the unknown serum was determined by the following equation:

$$\frac{\text{O.D. unknown - O.D. control}}{\text{O.D. standard - O.D. of blank}} \times 5$$

The product is the number of milligrams of β-naphthylamine liberated per 100 ml. serum per hour.

(2) Inhibitor Experiments

The effect of inhibition on the enzymatic splitting of L-Cystine-di- β-naphthylamide was investigated by incubation with sera from women 36 - 40 weeks pregnant. The enzymatic activity of the serum was determined as previously. In addition, 0.6 ml. of the serum was pre-incubated with the inhibitor solution (0.9 ml.) for 15 minutes at 37°C. The same inhibitors described in Chapter II were employed in a concentration of $5/3 \times 10^{-3}$M. The final concentration of the inhibitors in the mixture was thus 10^{-3}M, except in the case of E.D.T.A., where a final concentration of 10^{-2}M was aimed at. Veronal buffer (3.0 ml.) was then added and the enzymatic activity was determined as before.

The extent of inhibition was calculated from the formula:

$$\frac{a - b}{a} \times 100$$

where a is the enzymatic activity of a serum, and b is the residual enzymatic activity of the same serum in presence

of a specific inhibitor.

(3) Paper Electrophoresis of Pregnancy Sera

The separation of the serum proteins was carried out under the following conditions:

A volume of 0.1 ml. of late pregnancy serum was applied in a linear fashion to each of two cellulose acetate paper strips (Oxoid). Electrophoresis was carried out in an Eel X 118 electrophoresis apparatus in veronal buffer, pH 7.9, ionic strength 0.05 M. Through each strip a current of 0.3 m. amp. per cm. was run for approximately 17 hours to obtain a separation of 12 - 15 cms.

One of the strips was then dried in a hot air oven (ten minutes at 80°C) and then stained for the various protein bands with lissamine green (I.C.I. product S.F. 150, 0.3% in 15% acetic acid in tap water) as described by Gorringe, 1957. This strip was kept as a marker.

The other paper was cut up into 1 cm. strips while still wet. The strips were then carefully transferred to small test tubes containing 1.0 ml. of a mixture of veronal buffer (pH 7.9, ionic strength 0.05) and distilled water (2:1). The strips were completely submerged and allowed to stand for 24 hours in the refrigerator.

To each of the eluates 0.25 ml. of substrate solution was then added, and the mixtures incubated for 24 hours at 37°C. The reaction was finally stopped by adding 0.75 ml. of 10 per cent trichloracetic acid. The tubes were then centrifuged and the β-naphthylamine content of 1.0 ml. of the supernatant estimated. The recoveries in the various

fractions were calculated in terms of mgm. β-naphthylamine per 100 ml. serum per hour, and compared with the total enzyme activity of the unfractionated serum.

(4) Ammonium Sulphate Fractionation of Pregnancy Sera

This was carried out by adding 2 ml. of a solution of saturated ammonium sulphate in water to an equal volume of late pregnancy serum of known cystine aminopeptidase activity. After adequate mixing, the tube was centrifuged at 2000 r.p.m. for 10 minutes. The clear supernatant was decanted into a separate tube and dialysed against distilled water for 3 hours. The protein deposit was washed twice in two changes of 10 ml. distilled water, and finally taken up in 0.9% NcCl solution. The final volumes of both the supernatant and deposit solutions were adjusted to a volume of 4.0 ml. by 0.9% NaCl solution. The enzymatic activity of both solutions was estimated and compared to the activity of the unfractionated serum after correction for the dilution.

(5) Chemical Estimation of Enzymatic Activity on S-Benzyl-Cysteine- β-Naphthylamide Substrate

The method described by Tuppy (1965) was employed with minor modifications.

Reagents

Substrate Solution: S-Benzyl-Cysteine- β-Naphthylamide, 168 mg., was dissolved in 50 ml. of 1:1 mixture of 50% methylcellusolv (2 methoxy-ethanol), and 0.01 N HCl (v/v). This substrate was obtained from Professor H. Tuppy.

Buffer

Tris-HCl buffer 0.1 M, pH 7.9 (page 45)

Standard Solution of β-naphthylamine containing 2 ugm/2.9 ml. 0.01 N NaOH. This was freshly prepared every week by dilution (1:200) of a stock solution containing 136.6 mg. β-naphthylamine per litre 0.01 N NaOH.

Other Reagents

Methylcellusolv 50% in water (v/v)

NaOH 0.01 N

Method

Estimations were carried out in small tubes 10 x 75 mm. Duplicate tubes were used for the test together with a zero time control, a blank and a standard.

The incubation mixture consisted of the following:

Buffer	50 µl.
Serum	10 µl.
Distilled water	10 µl.
Methylcellusolv	20 µl.

The five tubes were then stood in a water bath at 37°C for ten minutes. The first tube was used as a zero time control, and into it was pipetted 10 µl. of substrate solution followed immediately by 2.9 ml. of NaOH. The next two tubes (duplicates) were mixed with substrate solution (10 µl.) and incubated for 10 minutes in the water bath at 37°C. The enzymatic reaction was then stopped by adding 2.9 ml. of NaOH solution.

The remaining two tubes served as a standard and

blank. The standard was prepared by adding 2.9 ml. of β-naphthylamine solution. The blank was prepared by adding 2.9 ml. of NaOH.

The β-naphthylamine content of the reaction mixture was then determined fluorimetrically. The maximum activation of this compound is at 335 mμ. Using a Locarte single sided fluorimeter MK 4 with a mercury arc lamp, LF 1 filter on the primary side (range of transmittance 254-400 mμ) and a colourless gelatine film on the secondary side, a standard reference curve was prepared for β-naphthylamine in the range 0.1 - 2 μgm/3 ml. 0.01 N NaOH as seen in Fig. 23.

The blank was set to zero and the standard to a reading of 100. The percentage fluorescence of the unknown incubation mixture was read. After subtracting the reading for the zero time control, the β-naphthylamine content was determined by referring to the standard curve.

The enzymatic activity was calculated as the number of mgs. β-naphthylamine released by 100 ml. serum per hour from the formula:

β-naphthylamine content of incubation mixture (in μgm) x 6 x $\frac{100}{100}$

Results

1 Kinetics of Enzymatic Splitting of L-Cystine-di- -naphthylamide by Sera of Women in Late Pregnancy

The standard reference curve for β-naphthylamine is shown in Fig. 24. The relationship between the colour

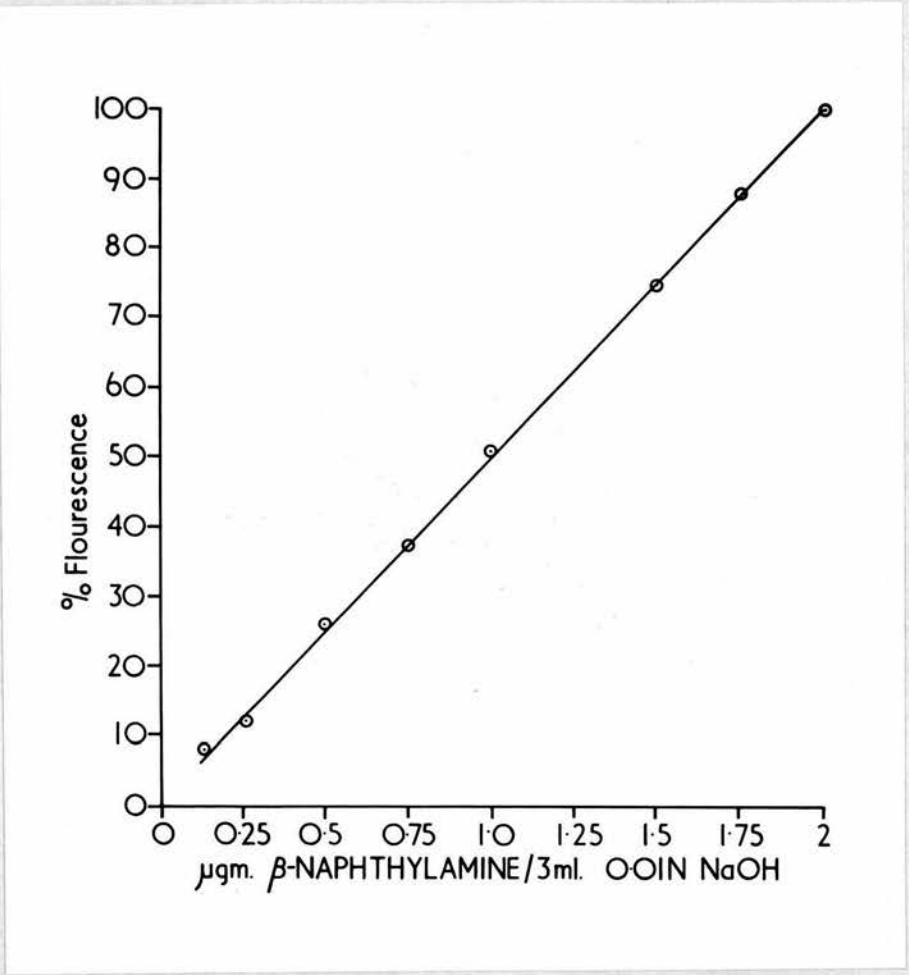


Figure 23 Reference Curve for Fluorimetric Estimation of β-naphthylamine. High Standard Contains 2 µgm. β-naphthylamine/3 ml. 0.01 N NaOH. Excitation at 335 mµ. Percentage Fluorescence Read at 410 mµ.

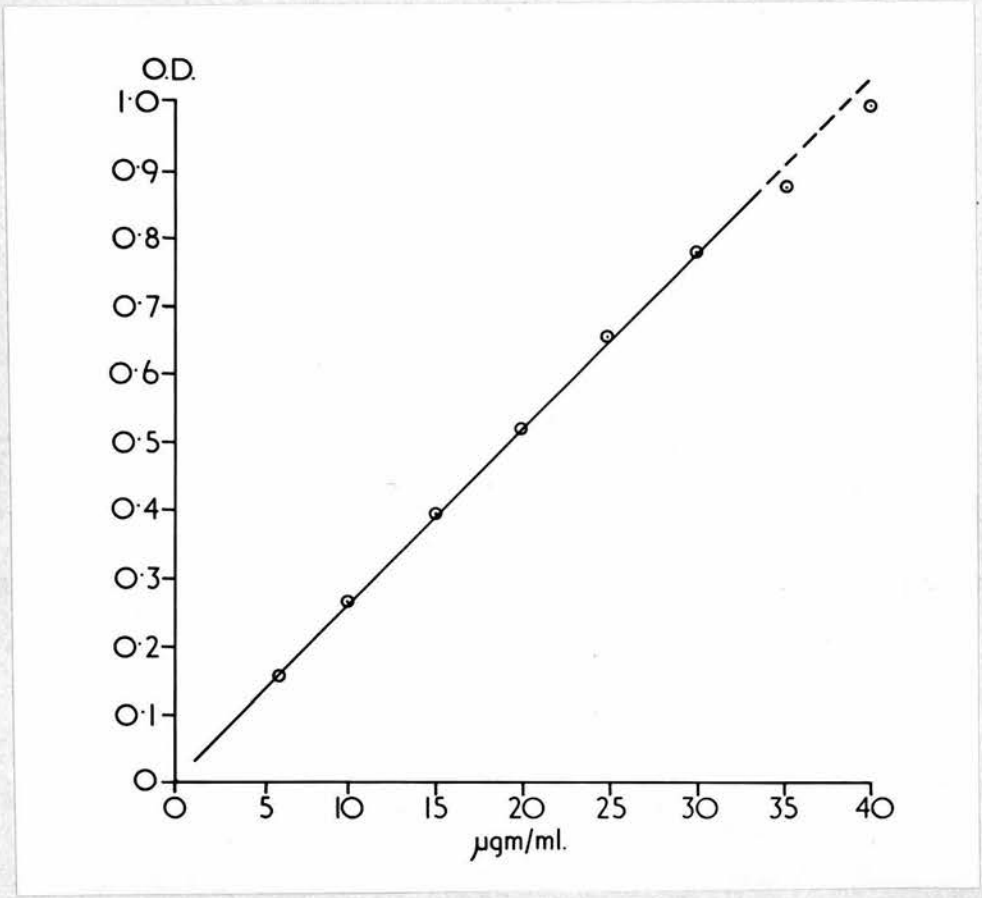


Figure 24 Reference Curve for Spectrophotometric Estimation of β-naphthylamine. The Optical Density (O.D.) is Plotted Against the Concentration of β-naphthylamine in µgms.

density (expressed as optical density: O.D.) and the β-naphthylamine concentration is linear over a wide range and up to a reading of O.D. 0.8. The final concentration of β-naphthylamine in the standards run in routine assays was 10 ugm. which gave an average O.D. of 0.26 (± 0.003).

The time course relationship of the enzymatic reaction under the standard conditions for incubation described on page 98 is shown in Fig. 25, where the β-naphthylamine release by specimens of serum from two subjects in late pregnancy were estimated at hourly intervals. A linear relationship is seen throughout the first four hours.

Fig. 26 shows the relationship between the serum concentration in the incubation mixture and the enzyme activity expressed as the O.D. of the β-naphthylamine released after four hours incubation. A linear relationship existed with serum concentration between 0.0 and 0.2 ml. serum per ml. of the incubation mixture. At higher serum concentrations, the β-naphthylamine release was relatively less. In the standard method a concentration of 0.1 ml. serum/ml. of the incubation mixture was regularly employed.

The effects of changes in pH are recorded in Fig. 27. Instead of veronal-HCl, a universal buffer (Teorell and Stenhagen's citrate-phosphate-borate buffer, 1938) was employed to allow investigation of enzyme activity in a wide range of pH changes (3 - 10). The maximum enzyme

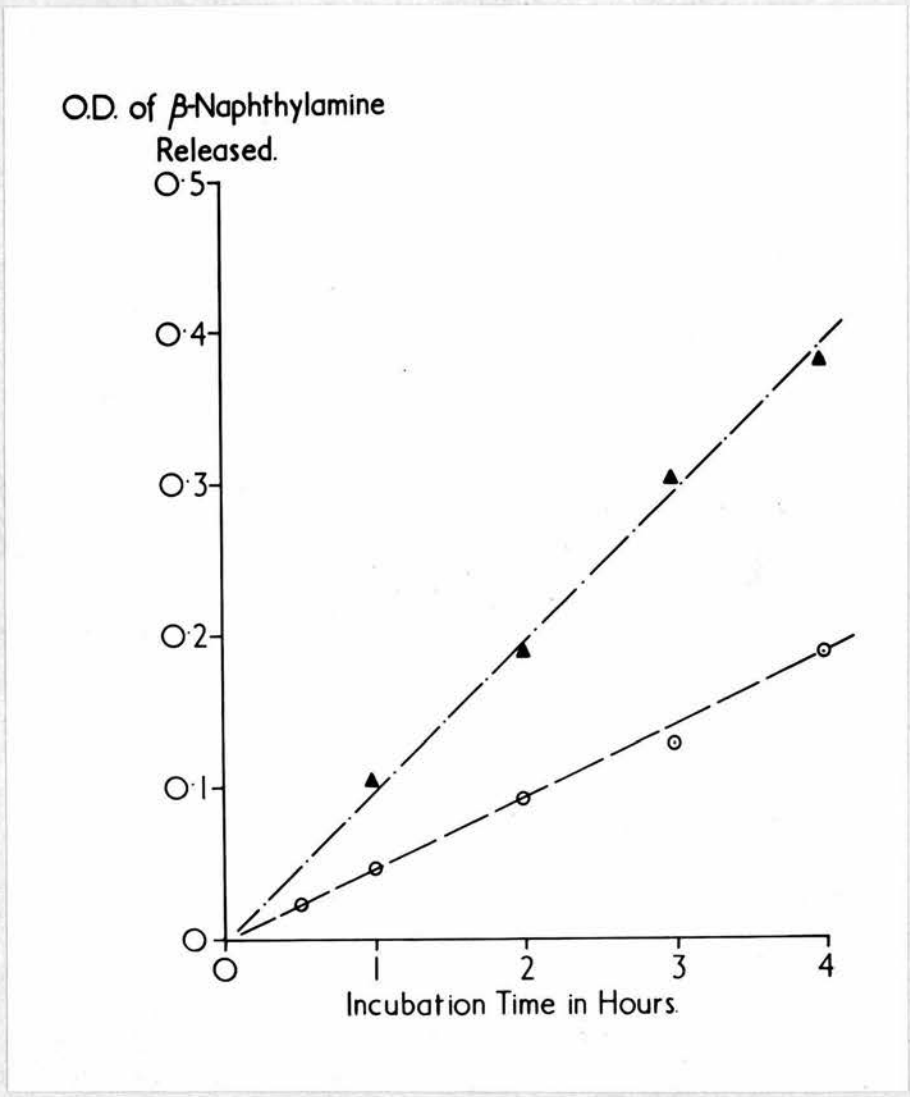


Figure 25 Progress Curves of the Enzymatic Splitting of L-Cystine-di-β-naphthylamide by Two Different Late Pregnancy Sera. Enzyme Activity Expressed as Optical Density (O.D.) of β-naphthylamine Released.

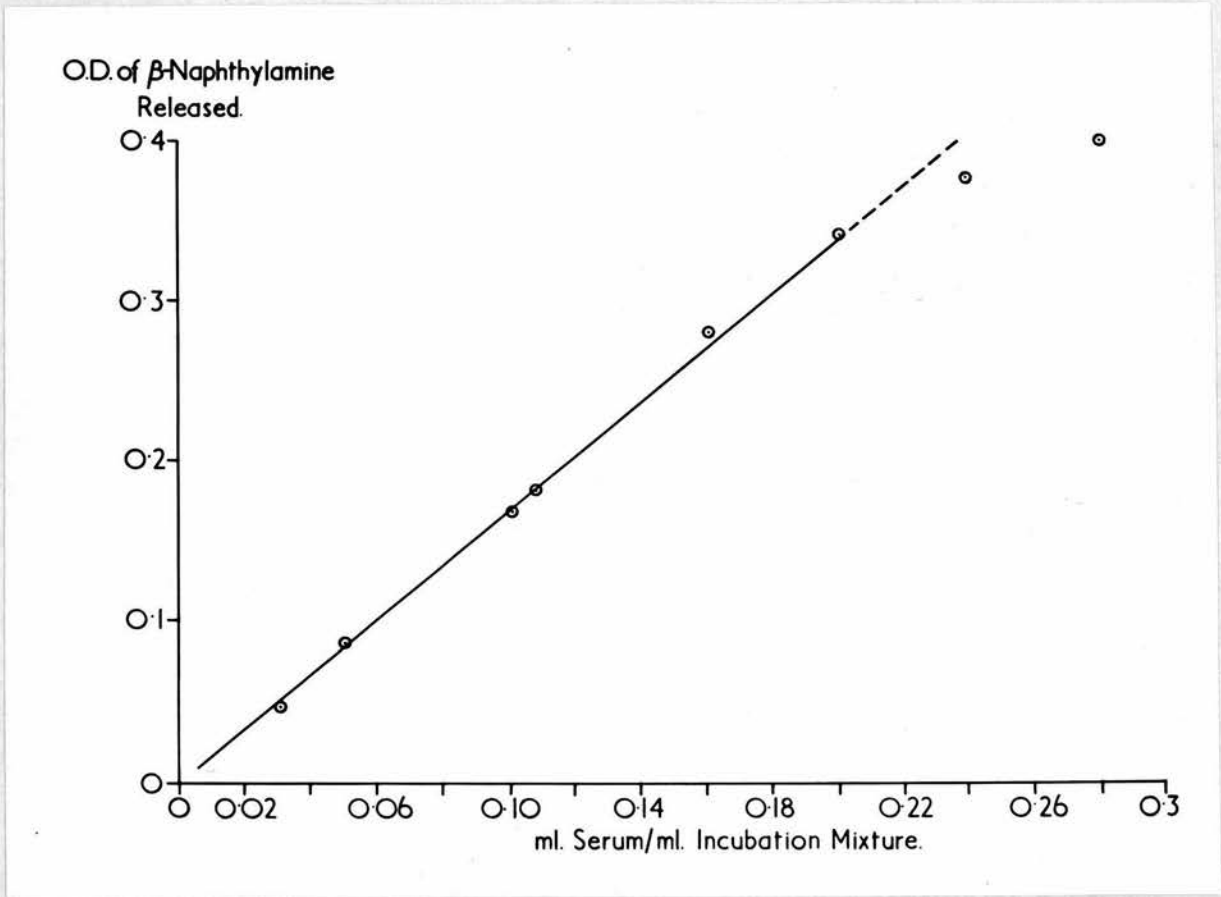


Figure 26 Effect of Changes in the Serum Concentration in the Incubation Mixture on the Enzymatic Splitting of L-Cystine-di-β-naphthylamide (Expressed as Optical Density "O.D." of β-naphthylamine Released).

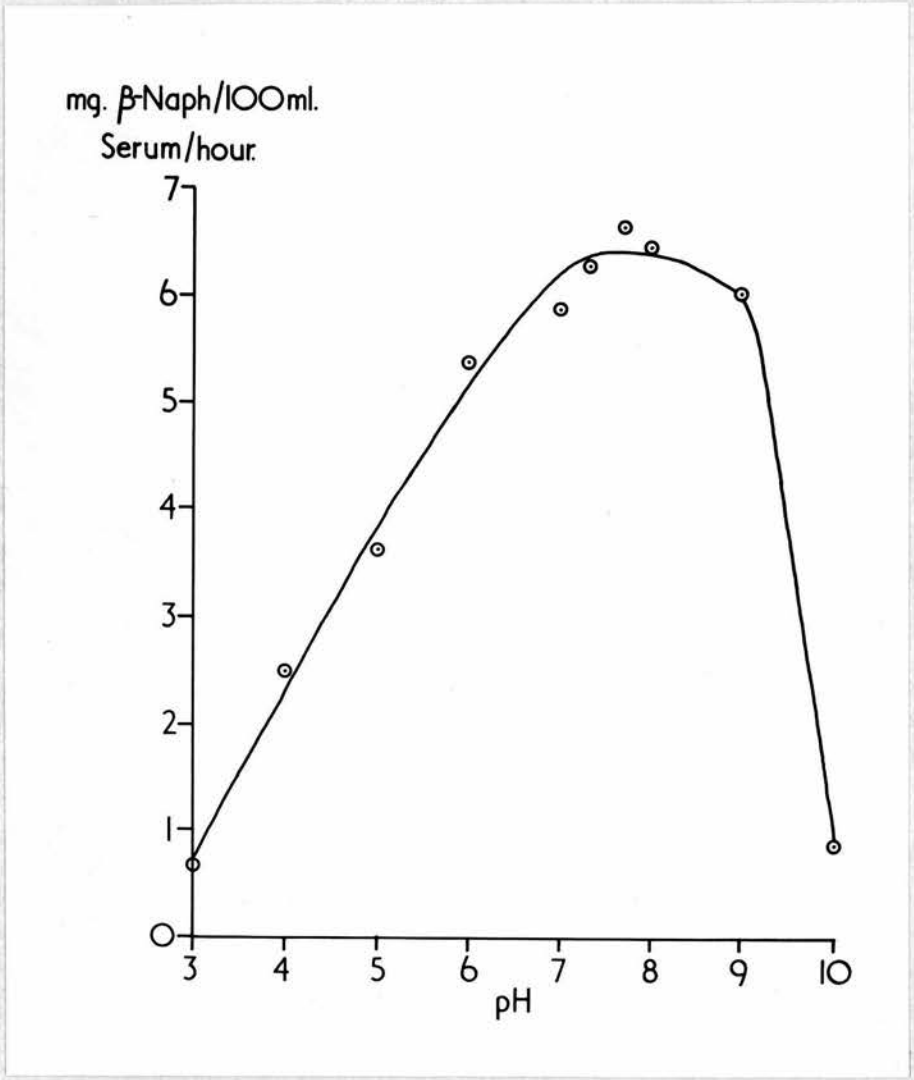


Figure 27 Effect of pH Changes in the Enzymatic Splitting of L-Cystine-di-β-naphthylamide by a Late Pregnancy Serum.

activity (expressed as O.D. of β-naphthylamine released) was found in the pH range 7.4 to 8.2. The loss of activity was more abrupt at higher pH readings than at lower ones.

2 "Cystine" Aminopeptidase Activity in Sera of Non-Pregnant Females

The enzyme levels were determined in the sera of 20 healthy female subjects. The enzyme activity varied between 0.17 and 0.59 mg. β-naphthylamine/100 ml. serum/hour, with an average of 0.30 (± 0.1) mg. Differences were not detected between enzyme levels in sera obtained in the follicular and luteal phases of the menstrual cycle.

3 "Cystine" Aminopeptidase Activity in Sera of Pregnant Animals

The sera of each of two pregnant rats, mice, cows, sheep and pigs were tested; all animals selected were in the last third of their gestational period. The results are shown in Table 16, and reveal only traces of activity. The levels were similar to those of non-pregnant human sera.

4 Effect of Inhibitors on Cystine Aminopeptidase Activity of Human Pregnancy Sera

The effect of inhibitors on the enzymatic splitting of the synthetic substrate L-Cystine-di-β-naphthylamide by late pregnancy sera is shown in Table 17, which reveals a similar pattern to their effect on the enzymatic splitting of oxytocin. Substantial inhibition was produced by Cu^{++}, Ag^{+}, Zn^{++}, and the metal-chelating agents 8-hydroxy-

TABLE 16

Source of Serum	Gestation Period	No. of Observations	Enzymatic Activity
Non-Pregnant Females	-	20	0.30 (± 0.10)
Pregnant Females	36-40 w	40	4.80 (± 0.12)
Pregnant Sheep	21-22 w	2	0.76
Pregnant Sow	11-12 w	2	0.72
Pregnant Cow	31-32 w	2	0.53
Pregnant Rat	2+ w	2	0.48
Pregnant Mouse	2^{+} w	2	0.32

The Distribution of Cystine Aminopeptidase Activity in Human and Animal Sera Expressed as mg. β-naph./100 ml. Serum/Hour

TABLE 17

Inhibitor 10^{-3}M	% Inhibition	
	Cystine Aminopeptidase	Oxytocinase
Ag^{+}	74.0	55 - 92
Cu^{++}	54.0	74 - 100
Fe^{++}	0.0	0.0
Zn^{++}	54.5	46-76
Iodacetamide	0.0	0.0
p.Chlormerc. benz.	0.0	0.0
E.D.T.A. (10^{-2}M)	91.0	46 - 100
8-Hydroxy-quinol.	42.0	48 - 53

Comparison of the Effect of Inhibitors on the Enzymatic Splitting of Oxytocin and L-Cystine-di-β-naphthylamide by Late Pregnancy Sera

quinoline and E.D.T.A. Fe^{++} and sulphlydryl inhibitors had very little effect. Similar results were reported by Tuppy and Wintersberger (1960).

5 Recovery and Distribution of "Cystine" Aminopeptidase Activity after Ammonium Sulphate Fractionation

Four late pregnancy sera were investigated. The results are shown in Table 18. After half saturation with ammonium sulphate, an average of 77.5 per cent of the enzyme activity was recovered in the supernatant, and 6.0 per cent in the precipitate. The average total recovery was 83.5 per cent. A similar observation reported by Werle and Semm (1956) on "oxytocinase" activity.

6 Recovery and Distribution of "Cystine" Aminopeptidase Activity after Paper Electrophoresis

The maximum enzyme activity in sera of pregnant women was found on the cathode side of the α_1-globulin fraction, with a considerable overlap both into α_1 and α_2 globulin bands (Fig. 28 and Table 19). The total recovery in three consecutive experiments averaged 31.4 per cent of the original activity in the sera. No enzymatic activity was recovered in the albumin, β or γ globulin fractions.

The results indicated that the enzyme did not occupy a sharply defined band on paper electrophoresis, but was recovered in the α_1 globulin and α_2 globulin bands, and in the zone in between. Werle and Semm (1956) reported a similar percentage recovery of oxytocinase activity after

TABLE 18

Enzymetic Activity Expressed as O.D. of Naph. Released

Unfractionated Serum	Supernatant	Precipitate	Recovery
0.234	0.244	0.024	0.268
0.200	0.157	0.004	0.161
0.260	0.154	0.012	0.166
0.175	0.120	0.014	0.134
0.869	0.675	0.054	0.729
100%	77.5%	6.0%	83.5%

Distribution of Pregnancy Serum Cystine Aminopeptidase Following 50% Ammonium Sulphate Precipitation. The Values for the Activity in Supernatant and Precipitate were corrected for the Dilution

TABLE 19

Protein Fraction	Cystine Aminopeptidase Activity				Average % Recovery
	Exp. 1	Exp. 2	Exp. 3	Sum	
Albumin Band	0.10	0.07	0.07	0.24	1.9
α_1 Globulin Band	0.14	0.30	0.50	0.94	7.6
$\alpha_1 - \alpha_2$ Globulin Interval	0.43	0.63	0.70	1.76	14.3
α_2 Globulin Band	0.23	0.30	0.20	0.73	5.9
β and γ Globulin Bands	0.10	0.04	0.07	0.21	1.6
All Fractions	1.00	1.34	1.54	3.88	31.4
Unfractionated Serum	3.15	4.21	5.00	12.36	100

Fractionation of Late Pregnancy Sera by Paper Electrophoresis. Enzyme Activity Expressed as mg. β-naphthylamine/100 ml. serum/hour

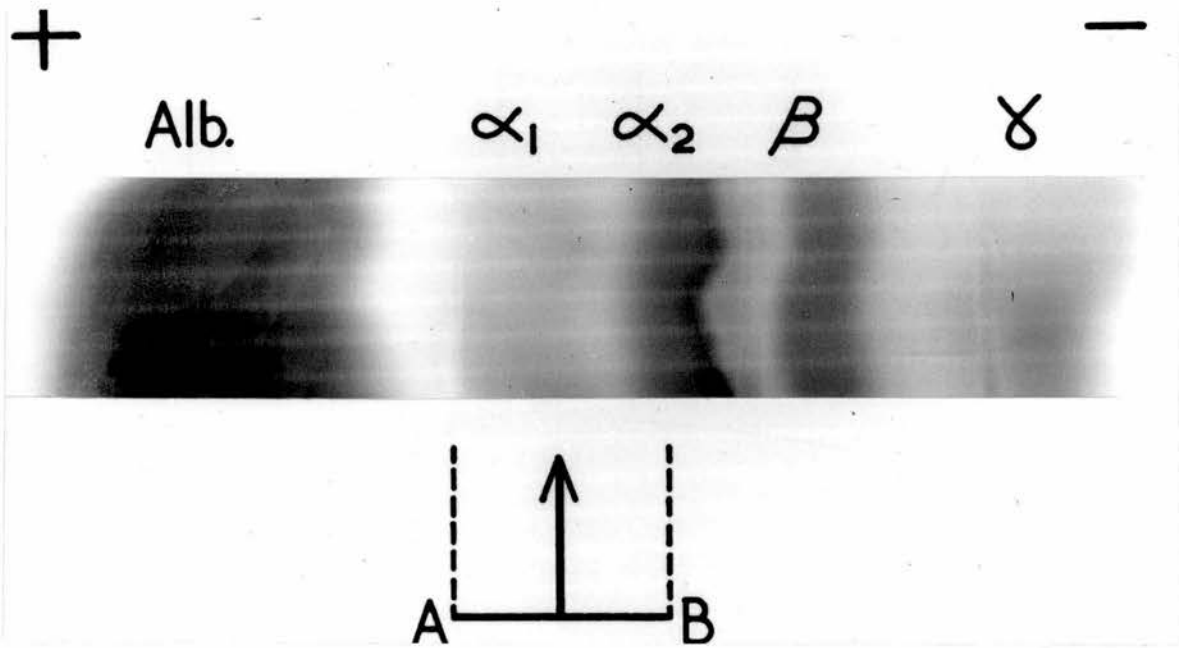


Figure 28 Electrophoresis of a Late Pregnancy Serum on Cellulose Acetate Paper, Showing the Various Protein Bands, the Point of Maximum Cystine Aminopeptidase Activity (marked with an arrow), and the Range of Enzyme Activity (marked with the straight line AB).

continuous electrophoresis of pregnancy sera, with the highest activity in the α_1 globulin fraction. They however, reported lower, but substantial, activity in the albumin band.

7 Comparison of the Enzymatic Splitting of L-Cystine-di-β-naphthylamide and S-Benzyl-Cysteine-β-naphthylamide by Pregnancy Sera

Parallel estimations were carried out on the sera of 24 women in various stages of pregnancy. The results are shown in Fig. 29, which indicates a good correlation between the two methods. In individual sera, the S-Benzyl-Cysteine substrate was split approximately 10 times faster than L-Cystine-di-β-naphthylamide.

To test the specificity of the two substrates for cleavage by early pregnancy sera (as opposed to non-pregnancy sera) and their possible use as a pregnancy test, Table 20 shows the average reading for 10 non-pregnancy sera (B) and 15 serum samples from patients in the first 10 weeks of gestation (A). The ratio A/B is nearly the same for both substrates.

Conclusions

1 The enzymatic splitting of the synthetic compound L-Cystine-di-β-naphthylamide showed the same general distribution in human sera and in the sera of pregnant animals as oxytocinase. The enzyme responsible in human pregnancy sera occupied the same position after electro-

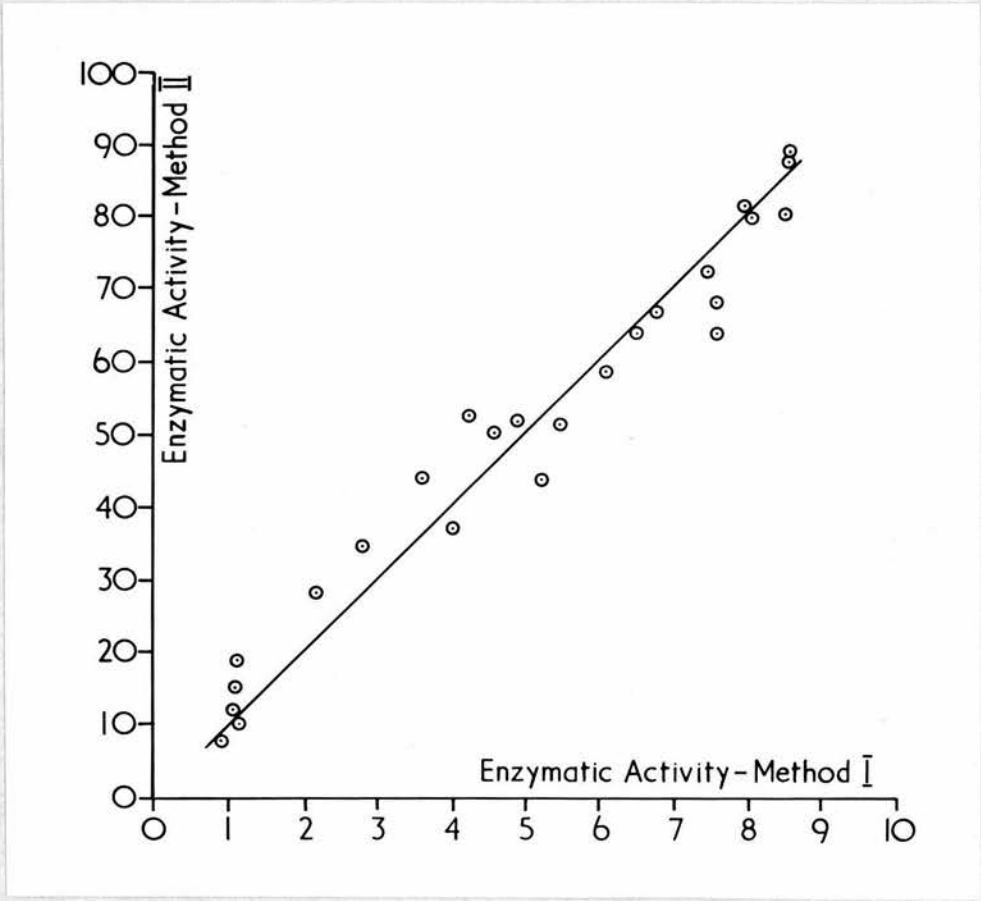


Figure 29 Comparison between the Enzymatic Splitting of L-Cystine-di-β-naphthylamide (Method I) and S-Benzyl Cysteinyl-β-naphthylamide (Method II) by Sera of Women in the Various Stages of Pregnancy. All Results are Expressed as mg. β-naphthylamine/100 ml. Serum/Hour.

TABLE 20

Source of Serum	No. of Observ.	Enzymatic Activity Method I	Method II
Non-Pregnant (B)	10	0.34 (0.22-0.62)	3.80 (2.16-7.20)
Pregnant 10 w. (A)	15	0.58 (0.32-0.8)	6.42 (3.60-9.30)
	Ratio A/B	1.70	1.69

Comparison of the Two Chemical Methods for Oxytocinase Estimation in the Diagnosis of Early Pregnancy. Enzymatic Activities are Expressed as mg. β-naphthylamine/100 ml. Serum/Hour Released from L-Cystine-di- β-naphthylamide (Method I) and S. Benz. Cysteine- β-naph. (Method II).

phoresis and ammonium sulphate precipitation as oxytocinase and also had similar properties when tested by various inhibitors. Thus there was every reason to accept cystine aminopeptidase activity as a valid estimate of oxytocinase in human pregnancy sera.

2 The newer synthetic substrate, S-Benzyl-Cysteine-β-naphthylamide was split more readily by pregnancy sera than L-Cystine-di-β-naphthylamide and allowed quicker estimation of enzymatic activity in micro litre volumes of sera. It was not, however, a better substrate for the diagnosis of early pregnancy.

E X P E R I M E N T A L

P A R T

C H A P T E R V

CLINICAL STUDIES ON PREGNANCY SERUM OXYTOCINASE

In this part of the work, oxytocinase activity was investigated by the chemical method in the sera of women at the various stages of normal pregnancy, labour and the puerperium. Estimations were also carried out in the various pathological conditions of pregnancy and labour.

To study the site of formation of the enzyme, the distribution of oxytocinase activity was investigated in the different products of gestation, as well as in vitro cultures of trophoblast. The serum levels were also tested in female volunteers receiving oral contraceptive tablets, which produce changes in the endometrium, not unlike the decidua of early pregnancy.

The possible physiological role of the enzyme in regulating myometrial contractility was examined in patients receiving intravenous infusions of oxytocin in advanced pregnancy with the purpose of induction of labour.

Material and Methods

Blood samples were collected by venipuncture as described on page 46 .

Blood samples from volunteers on contraceptive tablets (Ovulen: Mestranol 0.1 mg. and Ethynodial Diacetate 1.0 mg.) were collected between the 15th and 20th day of the cyclic course.

Amniotic fluid samples were obtained by puncture of the hind membranes by a Drew Smyth catheter in patients in early normal labour, or those undergoing induction in late pregnancy.

Placental extracts were prepared by homogenising normal term placentae in saline (1:1) as described on page 57. The enzyme content of the placenta was calculated as twice that of the supernatant.

Cultures of human trophoblast were prepared by Dr. N. Rashad from the placentae of cases terminated by abdominal hysterotomy in mid-pregnancy, using the technique described by Harnden (1960) for tissue culture. Oxytocinase estimations were carried out on the supernatant culture medium after signs of growth had been observed microscopically. The culture medium was changed every 48 hours and serial assays were carried out for 3 weeks. A sample of the same culture medium (culture medium 199 + 20% AB serum) used in harvesting the tissue was employed as a control.

All enzymatic estimations were carried out on the synthetic substrate L-Cystine-di-β-naphthylamide as previously described on page 97. The results are expressed in mg. β-naphthylamine/100 ml. serum/hour.

Results

Serum Oxytocinase Levels in Normal Pregnancy

One hundred and twenty random determinations were carried out in normal pregnant females between four and forty weeks of gestation. The results are expressed in Table 21 and Fig. 30, and reveal a gradual rise of oxytocinase activity from the non-pregnancy level (0.3 ± 0.10) to an average value of 4.8 (± 1.2) mg. β-naphthylamine/100 ml. serum/hour near to term.

TABLE 21

Period of Gestation	No. of Observ-ations	Average Activity	Range
4^+ to 8 weeks	10	0.4	0.14-0.6
8^+ to 12 weeks	10	0.5	0.4 -0.9
12^+ to 16 weeks	10	0.7	0.5 -1.0
16^+ to 20 weeks	10	1.1	0.8 -1.5
20^+ to 24 weeks	10	1.2	0.9 -1.6
24^+ to 28 weeks	10	1.9	0.9 -3.0
28^+ to 32 weeks	10	3.1	1.4 -5.1
32^+ to 36 weeks	10	3.5	2.2 -6.5
36^+ to 41 weeks	40	4.8	2.8 -8.2

Normal Pregnancy Serum Oxytocinase Levels. All Values Expressed as mg. β-Naphthylamine/100 ml. Serum/Hour.

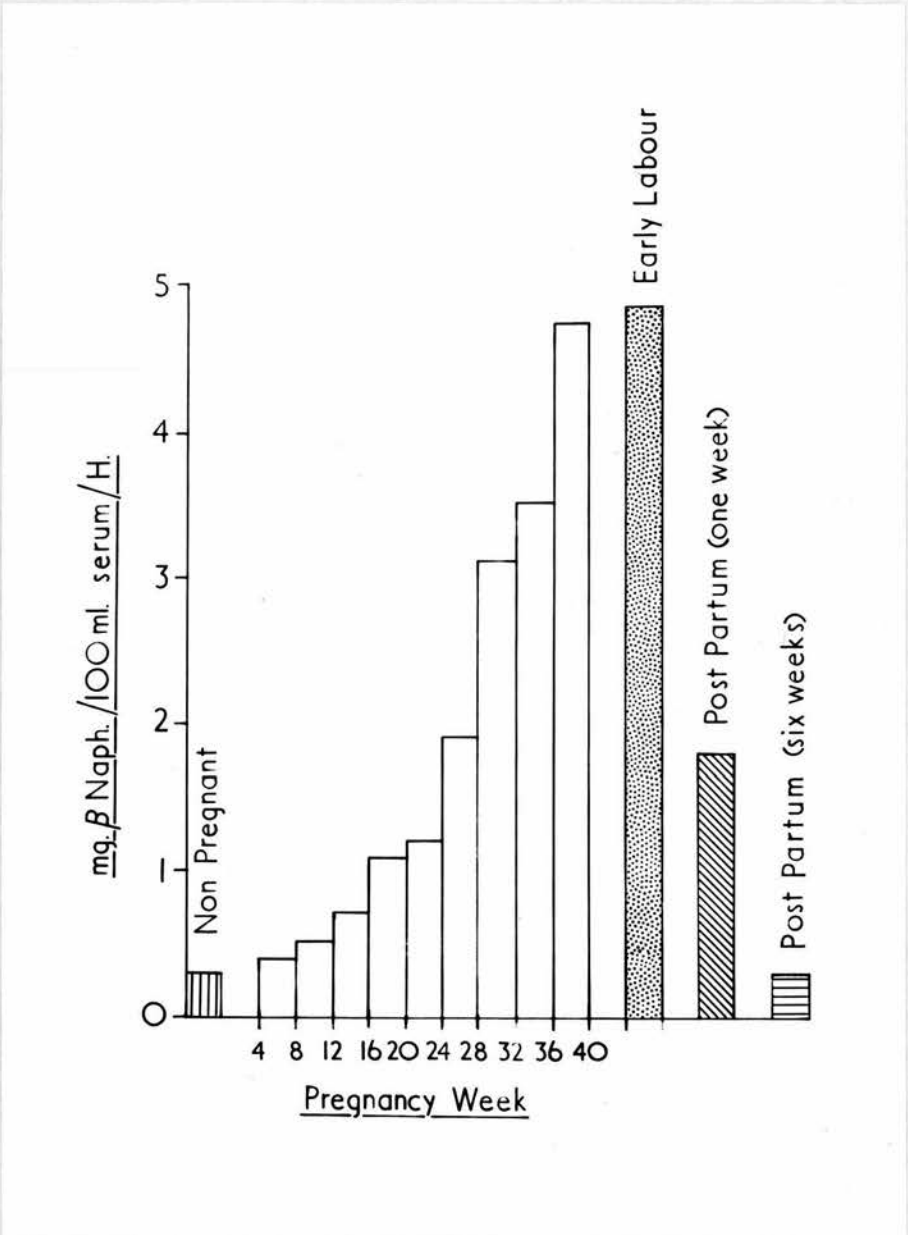


Figure 30 Average Values for Serum Oxytocinase at Various Stages of Pregnancy, in Labour, and during the Puerperium. Random Estimations.

Changes in Oxytocinase Levels in Labour and in the Puerperium

Fourteen normal pregnant subjects were followed by serial assays one to three weeks before term, in early labour and one and six weeks after delivery. The results are expressed in Table 22. The levels obtained in early labour showed only minor changes from the late pregnancy readings. A slight drop was observed in four patients, and did not exceed 15 per cent of the late pregnancy level.

The enzyme level dropped to approximately 40 per cent of the labour value within one week after delivery, and returned to non-pregnancy level by six weeks post partum.

Enzyme Levels in Abnormal Pregnancy and Labour

1 Threatened Abortion

Serum oxytocinase levels were examined in 15 patients admitted to the antenatal ward with a diagnosis of threatened abortion at 16 to 20 weeks of gestation. The enzyme levels varied between 0.7 and 1.6 units, with an average of 1.05 ($\pm$ 0.28) mg. β-naphthylamine/100 ml. serum/hour. The level is not statistically different from the normal reading for this period of gestation (1.10 $\pm$ 0.30 units) ($n = 23$, $t = 0.1$, $p > 0.25$).

Of these patients, four subsequently aborted within one week. Their serum oxytocinase levels (0.8, 1.3, 1.0 and 0.9 units) were again not different from the normal pregnancy readings.

TABLE 22

Name	Pregnancy	Labour early	Change	Post Partum 1 week	Post Partum 6 weeks
E.B.	4.5	5.0	+0.5	2.5	0.26
S.W.	4.2	3.8	-0.4	1.8	0.22
E.G.	3.9	4.7	+0.8	1.5	0.30
V.I.	5.0	5.0	0.0	2.4	0.50
M.R.	5.4	4.9	-0.5	2.2	0.28
H.S.	4.0	4.6	+0.6	2.2	0.24
G.B.	4.3	5.0	+0.7	1.5	0.25
E.B.	5.0	5.0	0.0	2.6	0.29
V.R.	3.0	2.6	-0.4	1.1	0.24
S.S.	4.8	5.1	+0.3	2.5	0.27
G.R.	4.5	4.7	+0.2	1.6	0.29
J.J.	2.8	2.9	+0.1	0.7	0.31
J.D.	4.3	5.5	+1.2	2.1	0.43
M.D.	4.6	4.3	-0.3	1.2	0.29
Average	4.4	4.6	+0.2	1.8	0.29

Serial Assays of Cystine Aminopeptidase Activity in Late Pregnancy, Labour and in the Puerperium. All Results are Expressed in mg. β-naphthylamine/100 ml. Serum/Hour.

2 Multiple Pregnancy

Fourteen cases of twins and one case of triplets were investigated between 34 and 40 weeks of pregnancy. The average reading was 6.9 units ($\pm$ 1.6) which is significantly higher than the late normal pregnancy reading of 4.8 $\pm$ 1.2 ($n = 55$, $t = 4.5$, $p < 0.001$).

3 Pre-eclampsia

Serum oxytocinase was determined in twenty pre-eclamptic patients between the 36th and 40th week of pregnancy. Only patients exhibiting albuminuria (more than a trace) with hypertension and/or oedema were selected for this study.

Enzyme levels in this group of patients ranged from 1.6 to 5.1 mg. β-naphthylamine/100 ml. serum/hour, with an average of 3.2 $\pm$ 0.9 mg. When compared with the average normal reading for this stage of gestation (4.8 $\pm$ 1.2) the difference is statistically highly significant ($n = 58$, $t = 5.3$, $p < 0.001$).

4 Vesicular Mole

The serum enzyme level in a case of vesicular mole diagnosed at 23 weeks of pregnancy was 1.2 units, which falls within the normal range for this stage of gestation (0.9 - 1.6). Following abdominal hysterotomy, the molar vesicles were expressed and the enzymatic activity of their fluid content was determined. A level of 1.0 mg. β-naphthylamine/100 ml. serum/hour was found, which is nearly identical with the maternal serum activity.

5 Uterine Inertia

Serum oxytocinase was estimated in ten patients with primary uterine inertia diagnosed clinically on the basis of weak infrequent uterine contractions and prolonged first stage of labour (+ 24 hours). The enzyme levels ranged from 4.1 to 7.2 mg. β-naphthylamine/100 ml. serum/hour (average 5.1 ± 0.9 mg.). The levels are not significantly different when compared to a level of 4.6 (± 0.7 units) found in the normal first stage of labour (n = 22, t = 1.8, p 0.05).

Distribution of the Enzyme in the Products of Gestation

1 Umbilical Cord Blood

Ten samples were investigated. The serum oxytocinase ranged from 0.20 to 0.63 mg. β-naphthylamine/100 ml. serum/hour, with an average of 0.4 mg. The difference from the non-pregnant serum reading is not statistically significant.

2 Amniotic Fluid

The enzyme levels were determined in four specimens. Only traces of activity (0.08 to 0.13 mg. β-naphthylamine/100 ml. serum/hour) were found.

3 Placenta

The enzyme content of eight full term placentae varied between 13.4 and 23.5 mg. β-naphthylamine/100 gm. wet placental tissue/hour, with an average of 19.0 (± 3.9) mg.

Uterine Blood Levels

Six samples were investigated. Their serum oxytocinase content was compared to the levels in systemic (arm) vein

blood taken simultaneously. The results are shown in Table 23, and reveal no significant differences. The small variations obtained are well within the range of error of the chemical procedure.

Oxytocinase Levels in the Sera of Subjects Receiving an Oral Contraceptive

Nine volunteers were examined. The details of their clinical histories and the serum enzyme activities are shown in Table 24. The average figure (0.31 ± 0.1 units) is not significantly different from the normal non-pregnant control value of 0.30 (± 0.1) mg. β.naphthylamine/100 ml. serum/hour.

Changes in Serum Oxytocinase Activity during Oxytocin Infusion

Changes in the level of the serum enzyme were tested in pregnant women requiring oxytocin infusion for induction of labour near to term. The membrane had been ruptured for 16 to 26 hours in all cases at the time of oxytocin infusion. Blood samples were obtained at the start of the oxytocin drip. In cases of succesful induction, a second specimen was collected at the time of stoppage of the drip, usually half an hour after delivery of the placenta. When operative delivery was undertaken, the second sample was obtained immediately before obstetrical intervention. The rate of oxytocin infusion was frequently adjusted according to the uterine response (assessed clinically) and varied between 1 - 16 mU of oxytocin/min.

The clinical details and the results are shown in

TABLE 23

Subject	Age	Parity	Gestation w.	Indication for C.S.	Oxytocinase Activity	
					Uterine Vein	Arm Vein
S.D.	29	1	36^{+}	Diabetes Mellitus, Previous Section	3.42	3.35
H.F.	35	6	35^{+}	Rh. Iso. Failed Induction	3.25	3.40
S.R.	32	0	40	Breech Presentation	5.40	5.34
M.N.	25	0	39^{+}	Breech. Contracted Pelvis	7.32	7.21
G.T.	30	0	42	Failed Induction for Post-Dates	6.40	6.52
I.S.	22	2	41	Unstable Lie, Previous Section	4.16	4.10

Serum Oxytocinase Levels in Uterine and Arm Vein Blood Obtained Simultaneously in Patients Undergoing Lower Segment Caesarean Section. All Infants were Live Born. Enzyme Activity Expressed as mg. β.naph./100 ml. Serum/Hour.

TABLE 24

Name	Age	Pariry	Last Delivery	L.M.P.	Months on Contraceptive Tablets	Oxytocinase
M.S.	34	4^{+2}	18 M	23 D	1 M	0.36
B.M.	32	4^{+0}	32 M	25 D	24 M	0.26
N.A.	38	4^{+0}	30 M	21 D	24 M	0.31
E.P.	29	2^{+1}	12 M	24 D	10 M	0.34
R.G.	33	2^{+0}	22 M	24 D	18 M	0.17
A.R.	21	0^{+0}	-	24 D	20 M	0.52
M.L.	28	2^{+1}	14 M	22 D	9 M	0.27
H.R.	25	3^{+0}	30 M	23 D	20 M	0.32
A.W.	42	2^{+0}	8 Y	21 D	4 Y	0.62
					Average	0.35
					S.D.	($\pm$0.10)

Serum Oxytocinase Levels of Patients on Oral Contraceptive Tablets (Ovulen). Results Expressed in mg. β-Naph./100 ml. Serum/Hour.

TABLE 25

Name	Age	Parity	Indication	Gestation w	Duration of Drip H	Oxytocin Dose U	Duration of Labour H	Outcome	Oxytocin -ase at start	Oxytocin -ase at end	% Change
M.G.	40	4^{+1}	A.P.H.	38	16.5	15.0	14.7	N.D.	6.1	6.1	0.0
M.E.	27	0	Hyper-tension	40^{+}	15.0	6.0	12.3	N.D.	5.4	5.6	+3.7
L.P.	17	0	Post-Dates	41^{+}	7.3	6.0	8.3	N.D.	4.8	4.8	0.0
M.C.	25	1	Rh. Iso.	38^{+}	5.8	6.0	2.3	N.D.	6.5	6.6	+1.7
J.B.	22	0	Post-Dates	42	10.0	4.0	9.1	N.D.	6.6	6.7	+1.7
M.B.	33	0^{+1}	Post-Dates	41^{+}	14.5	13.0	12.6	N.D.	5.2	5.2	0.0
M.D.	36	1	Rh. Iso.	40^{+}	16.5	8.0	9.5	N.D.	4.3	4.4	+2.3
W.B.	27	0	P.E.T.	39	13.0	6.0	6.3	N.D.	5.0	5.0	+4.0
J.N.	34	3	Post-Dates	42	6.0	2.0	4.3	N.D.	5.9	5.8	-1.7
S.L.	25	0	A.P.H.	37	13.0	2.5	8.5	N.D.	5.9	5.9	0.0
M.F.	31	4	Post-Dates	42	6.0	2.0	4.3	N.D.	6.8	6.5	-4.4
M.B.	29	0	P.E.T.	39	12.8	8.5	9.0	N.D.	5.2	5.1	-1.9
A.S.	30	0	Post-Dates	42	10.0	6.5	7.5	N.D.	5.9	6.0	+1.8
M.W.	28	2	P.E.T.	38	6.3	2.0	5.0	N.D.	3.4	3.4	0.0
S.S.	30	2	Rh. Iso.	40^{+}	16.0	14.0	12.3	N.D.	6.5	6.6	+1.7
A.C.	29	3	Post-Dates	42	8.3	2.5	7.0	N.D.	7.0	7.0	0.0

continued

TABLE 25 (cont.)

Name	Age	Parity	Indication	Gestation w	Duration of Drip H	Oxytocin Dose U	Duration of Labour H	Outcome	Oxytocin-ase at start	Oxytocin-ase at end	% Change
S.K.	30	2	Post-Dates	42	7.0	2.0	6.5	N.D.	8.0	8.0	0.0
M.R.	25	0	P.E.T.	37	8.0	2.0	7.3	N.D.	2.8	2.9	+3.5
M.P.	24	1^{+1}	P.E.T.	38^{+}	12.2	6.0	9.0	N.D.	4.2	4.2	0.0
M.J.	26	0	Post-Dates	42	16.0	8.0	13.5	N.D.	4.3	4.4	+2.4
J.B.	32	2^{+2}	Rh. Iso.	37	11.5	8.0	9.5	N.D.	4.2	4.0	-4.8
E.S.	29	1	A.P.H.	37	8.5	3.0	7.3	N.D.	4.9	4.9	0.0
22 Patients				Total	240.2	133.0	186.1		118.9	119.1	10.6
Prim.		46%		Mean	10.9	6.0	8.5		5.4	5.4	0.45
Parous		54%		S.D.	(± 3.8)	(± 3.9)	(± 3.1)		(±1.3)		

Serum Oxytocinase Estimations in Cases of Successful Induction by Oxytocin Drip, with Slight Changes in the Enzyme Levels

TABLE 26

Name	Age	Parity	Indication	Gestation w	Duration of Drip H	Oxytocin Dose U	Duration of Labour H	Outcome	Oxytocin-ase at start	Oxytocin-ase at end	% Change
M.C.	25	2	Cardiac II	40	5.3	2.0	3.5	N.D.	4.7	5.2	+10.6
C.D.	29	0	Post-Dates	41^{+}	10.6	6.0	9.5	N.D.	4.6	5.1	+10.9
M.C.	26	2	Rh. Iso.	39	11.5	14.0	3.0	N.D.	4.0	4.8	+20.0
E.C.	39	3	P.E.T.	39^{+}	12.3	4.5	8.0	N.D.	3.8	4.8	+27.0
P.T.	27	2	Post-Dates	42	12.5	7.5	16.3	N.D.	7.8	9.0	+15.4
H.M.	21	0	P.E.T.	39^{+}	9.5	2.0	7.3	N.D.	5.6	6.6	+18.0
M.M.	25	0	Post-Dates	41^{+}	9.0	4.0	7.1	N.D.	5.3	6.4	+20.8
C.K.	22	1	Post-Dates	41^{+}	9.5	6.0	7.6	N.D.	5.6	7.0	+25.0
F.S.	26	2	Rh. Iso.	37^{+}	8.3	3.0	6.5	N.D.	3.4	4.1	+20.6
J.R.	20	0	Post-Dates	41^{+}	5.5	2.0	5.0	N.D.	5.6	6.6	+18.0
G.C.	28	2^{+1}	Rh. Iso.	37	5.8	10.0	2.5	N.D.	4.6	5.6	+21.7
S.B.	24	0	Post-Dates	43	14.3	6.0	8.3	N.D.	5.9	7.0	+18.6
12 Patients				Total	114.1	67.0	74.6		60.9	72.2	226.6
Prim.		42%		Mean	9.5	5.6	6.2		5.1	6.0	18.8
Parous		58%		S.D.	($\pm$2.8)	($\pm$3.9)	($\pm$2.2)		($\pm$1.4)		

Serum Oxytocinase Estimations in Subjects Showing a Rise in the Enzyme Levels Following Oxytocin Infusion for Induction of Labour.

TABLE 27

Name	Age	Parity	Indication	Gestation w	Duration of Drip H	Dose U	Outcome	Oxytocinase at start	Oxytocinase at end
A.S.	30	0	Post-Dates	42	8.0	2.5	Forceps D.	5.9	6.0
E.N.	25	0	P.E.T.	39^{+}	5.8	1.5	Forceps D.	4.1	4.1
B.M.	22	0	Post-Dates	42	14.0	8.5	Forceps D.	5.6	5.6
B.B.	34	1	Post-Dates	42^{+}	8.0	6.5	C.S.	5.5	5.4
M.M.	29	0	P.E.T.	39^{+}	9.8	14.0	C.S.	4.0	4.0

Serum Oxytocinase Changes in Cases of Induction by Oxytocin Drip Requiring Obstetrical Interference

Tables 25, 26 and 27. The duration of labour recorded in these tables was the interval between the onset of regular painful uterine contractions to the end of the third stage of labour (expulsion of the placenta). Subjects who were in established labour before starting the oxytocin infusion were excluded from this study.

In the majority of patients (Table 25), no significant changes in serum oxytocinase were noted. The slight variations noted in this group were well within the range of error of the chemical assay procedure.

A significant increase of serum oxytocinase activity, more than 10% of the pre-infusion level, was noted in 12 cases of successful induction. All cases of failed induction (ending in Caesarean Section) did not show this rise.

Comparing the average values for the data shown in Tables 25 and 26 as regards the dose of oxytocin infused, duration of infusion, length of labour, and pre-infusion level of oxytocinase, the only significant difference is the duration of labour, which was shorter in patients who showed the rise in oxytocinase levels (18.8%), as compared to subjects who did not show this increase. The statistical analysis of the data is shown in Table 28.

In Fig. 31, the extent of rise in oxytocinase level is plotted against the dose received. No significant correlation can be seen. It may also be noted that subject M.G., who received the largest dose of oxytocin in this series, (15 U), did not show a rise in oxytocinase levels.

TABLE 28

Parameter	Group A	Group B	Statistical Analysis n	t	p
Rise in Serum Oxytocinase	0.45%	18.8%			
Dose of Oxytocin	5.6 (± 3.9)	6.0 (± 3.9)	32	0.29	> 0.25
Duration of Drip	9.5 (± 2.8)	10.9(± 3.8)	32	1.1	> 0.25
Duration of Labour	6.2 (± 2.2)	8.5(± 3.1)	32	2.3	< 0.05
Pre-infusion oxytocinase Levels	5.1 (± 1.4)	5.4(± 1.3)	32	0.9	> 0.25

Statistical Analysis of Data in Tables 25 (Group A) and 26 (Group B)

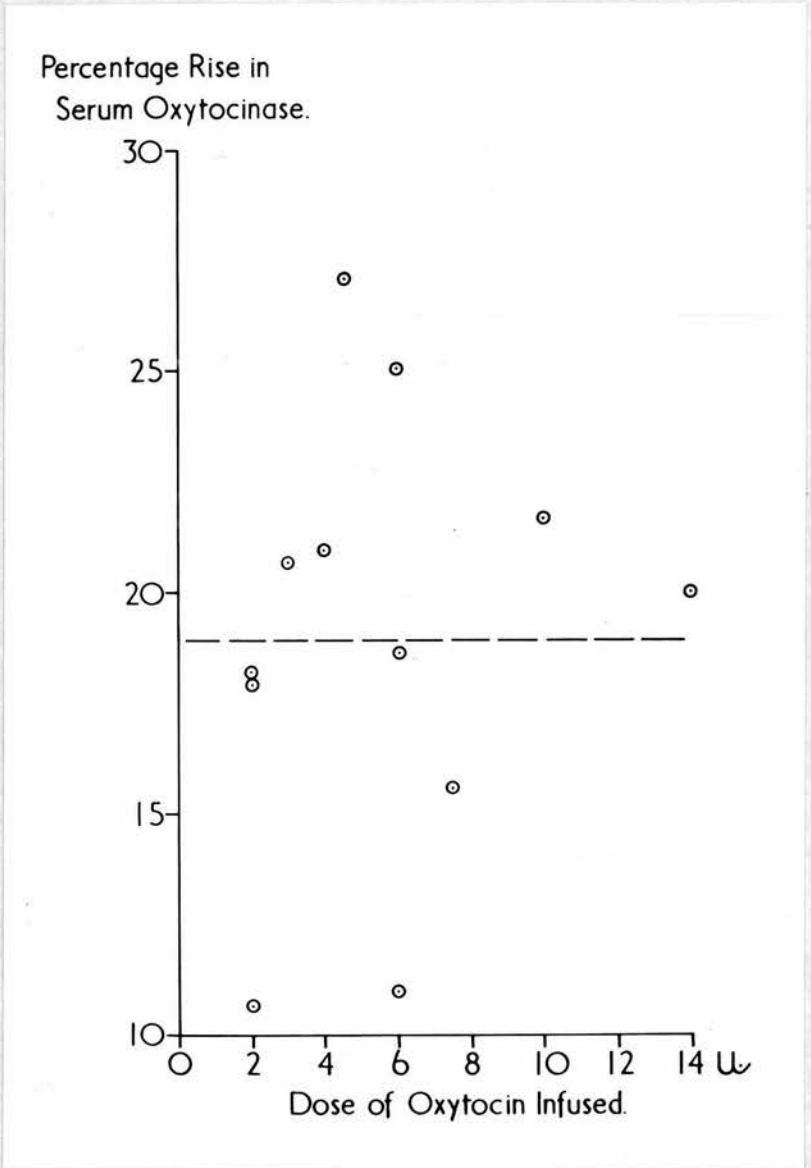


Figure 31 Relationship between Rise in Serum Oxytocinase and Dose of Oxytocin Infused.

Oxytocinase Production in Cultures of Trophoblast

Three cultures of trophoblast were investigated. The selection of tissue chosen from the placentae for incubation was done by micro-dissection, and the nature of the growing cells was confirmed by the microscopic appearance of the cells in the earlier phases of growth.

Controls showed a trace of cystine aminopeptidase activity of 0.08 to 0.13 units (probably due to their serum content). The three cultures of trophoblast were followed for 3 weeks. The levels recorded in 11 estimations varied between 0.08 and 0.12 mg. β.naphthylamine/100 ml. serum/hour.

Conclusions

1 Random chemical estimations of serum oxytocinase activity show a progressive rise throughout pregnancy to a level of 4.8 ($\pm$ 1.2) units near to term, with no significant change with the onset of labour. The enzyme levels decline slowly after delivery. Approximately 40% of the labour values are still present one week post partum.

2 The enzyme levels are significantly high in patients with multiple pregnancy, and significantly low in pre-eclamptic subjects. The enzyme levels in patients with threatened abortion, vesicular mole and uterine inertia in labour show no significant deviation from the normal.

3 Following oxytocin drip for the induction of

labour, a substantial rise of serum oxytocinase activity was noted in 12 out of 39 patients. The rise was not related to the outcome of the induction, nor to the dose received, but was associated with short labour.

4 The serum oxytocinase activity in uterine vein blood is similar to the systemic levels.

5 No substantial oxytocinase activity could be found in the sera of volunteers on oral contraceptive tablets, nor in "in vitro" cultures of trophoblast.

GENERAL DISCUSSION

THE MECHANISM OF INACTIVATION OF OXYTOCIN AND VASOPRESSIN BY PREGNANCY SERA

The present study indicates that the enzymatic degradation of pure synthetic oxytocin by sera of pregnant women involves cleavage of the peptide bond between the amino-terminal half-cystine residue (position 1) and the tyrosine residue (position 2). The resultant open chain compound has different R_f values in both Bu-A solvent (0.84) and Me-P solvent (0.44) as compared with the parent hormone (R_f 0.43 and 0.83 respectively). This open chain polypeptide is not available for direct comparison. Its nature, however, could be assessed indirectly by oxidation, which resulted in two components, one of which could be identified as L-cysteic acid.

Tuppy and Nesvadba (1957), reported a similar observation. They, however, gave no experimental details and did not define the R_f values of the enzymatic degradation product of oxytocin or its two components obtained by subsequent oxidation.

In the present work, it also appears that lysine vasopressin is cleaved by pregnancy sera by an analogous mechanism. As speculated by Tuppy (1960), this is not surprising in view of the fact that the two hormones have the same amino acid sequence in this part of the molecule, the difference being only in positions 3 and 8. Experimentally, the open chain degradation enzymatic product of lysine vasopressin has an R_f 0.22 in Bu-A and 0.44 in Me-P, in contrast with the parent hormone which has R_f 0.09 and 0.79 in these two solvents respectively. Subsequent oxi-

dation leads to formation of two components, of which one was identified as L-cysteic acid. It must be pointed out that in our second solvent (Me-P) oxytocin and lysine vasopressin, as well as their enzymatic degradation and oxidation products, have almost the same R_f values. Unfortunately, I have been unable to obtain human (arginine) vasopressin in a suitable concentration for similar experiments, but it would be surprising if the results on this chemically similar hormone prove any different.

In view of this identical process of cleavage, it is difficult to accept that oxytocin and vasopressin are inactivated by two different enzyme systems in pregnancy sera. This opinion was held by Werle (1960)with the following arguments, which are based on the results of biological assay experiments of serum oxytocinase and vasopressinase activity.

(a) Non-pregnancy female sera and sera of men have substantial "vasopressinase" activity, but are devoid of oxytocin-splitting enzymes. As pointed out by Tuppy (1960) this may be due to the existence of an unspecific aminopeptidase in the sera which is capable of attacking the vasopressin molecule in areas not represented in oxytocin, e.g. arginine (or lysine)-glycin-amide bond. This does not rule out the present argument that the specific pregnancy enzyme is capable of inactivating both molecules by an identical mechanism.

(b) Pregnancy serum oxytocinase and vasopressinase are affected differently by various enzyme inhibitors. Werle's short report (1960) contains no experimental details, but it is possible that the results of assay of residual oxytocin and vasopressin were affected by the influence of inhibitors on the assay preparation. One of the inhibitors employed (p-Chlormercuric benzoate) was found by Hooper (1959) to interfere with the assay of vasopressin on the blood pressure of rats.

There are several observations in the literature to confirm this mechanism of action of pregnancy serum oxytocinase, as first proposed by Tuppy and Nesvadba (1957) and confirmed in the present study.

(1) Stoklaska and Wintersberger (1959) reported that purified pregnancy serum oxytocinase preparations inactivated both oxytocin and vasopressin at similar rates.

(2) Berankova et al (1961) incubated late pregnancy sera with synthetic peptide compounds representing fragments of the oxytocin molecule and assessed their rate of cleavage by ninhydrin titration of free amino groups and by paper chromatography. The results indicate that pregnancy sera hydrolyse the compound S-benzylcysteinyl-tyrosine (amino acid sequence 1 and 2 of the oxytocin molecule), but do not split S-benzylcysteinyl-prolyl-leucyl-glycine amide (amino acid sequence 6 to 9 of the oxytocin molecule). By extending the range of substrates

they characterised the pregnancy enzyme as a specific aminopeptidase which is distinct from tissue oxytocinase, leucine aminopeptidase, carboxypeptidase, chymotrypsin, dipeptidase and tripeptidase. These results were subsequently confirmed by Cihar et al (1961) on a purified pregnancy serum oxytocinase preparation.

(3) Glendening et al (1965) localised oxytocinase activity in two specific bands by vertical starch gel electrophoresis. The enzyme eluted from both bands was capable of inactivating both oxytocin and vasopressin.

(4) Golubow et al (1963) reported that the potent synthetic analogue 1-Desamino-oxytocin is not inactivated by pregnancy sera. This compound has exactly the same structure as oxytocin except for the absence of a free amino group in position 1, which thus seems to be essential for the splitting of the molecule by the specific pregnancy serum enzyme which acts as an "amino"-peptidase.

PURIFICATION, PHYSICO-CHEMICAL PROPERTIES AND SUBSTRATE SPECIFICITY OF PREGNANCY SERUM OXYTOCINASE

Purification of the enzyme has been attempted by Tuppy and Wintersberger (1960). Employing "retroplacental serum" as a starting material, they achieved an overall purification of 2300 - 4500 fold as estimated by the specific activity per unit weight of protein. Purification involved ammonium sulphate and rivanol precipitation, removal of contaminants with bentonite and repeated column

chromatography on hydroxylapatite. The preparation appeared as a single band in horizontal starch gel electrophoresis.

In the present study, paper electrophoresis indicates that enzymatic activity is recovered both in the α_1 and α_2 globulin fractions and the zone in between. Similarly, Page et al (1961) reported that in vertical starch gel electrophoresis of pregnancy sera, oxytocinase could be recovered in two distinct bands. Cihar et al (1961) also reported that during fractionation of pregnancy sera by ethanol (according to the methods of Cohn et al, 1950), oxytocinase activity was distributed between the albumin and globulin fractions. The two forms of oxytocinase had different physico-chemical properties, but the same substrate specificity and inhibition properties. They suggested that the two forms may differ in their content of lipids or sugars, but have the same catalytic nucleus.

Tuppy et al (1963) confirmed that the enzyme is a glycoprotein. Following incubation of a purified preparation with neuraminidase, neuramic acid was released. The removal of the acidic sugar molecules altered the electrophoretic mobility of the enzyme which still retained its oxytocinase activity. Tuppy and Wintersberger (1964) suggested that a similar change might occur during the fractionation processes, and could account for the occurrence of two forms of oxytocinase reported by other workers.

The present study indicates that the enzyme is a

simple aminopeptidase. Tuppy and Nesvadba (1957) reported similar findings, and suggested that pregnancy serum oxytocinase is a specific "cystine" aminopeptidase acting on molecules, which like oxytocin and vasopressin, possess amino-terminal half-cystine residues. Subsequent studies on purified preparations confirmed that synthetic substrates of such configuration are typical substrates for the enzyme. These include

L-Cystine-di-β-naphthylamide (Tuppy and Nesvadba, 1957)

S-Benzyl-cysteinyl-β-naphthylamide (Tuppy, 1964)

L-Cystine-dityrosinamide (Wintersberger et al, 1960)

S-Benzyl-cysteinyl-tyrosine (Berankova et al, 1961)

In addition, the enzyme is also capable of splitting a wide variety of amino acid-β-naphthylamide compounds which lack this specific configuration (Tuppy and Wintersberger, 1960). L-leucine-β-naphthylamide is split even more readily that the cystine derivative. The label "Cystine" aminopeptidase is thus only useful to indicate the type of substrate usually employed to estimate oxytocinase, but in no way embraces its amino acid substrate specificity.

The only other aminopeptidase that has been purified and extensively studied is "Leucine" aminopeptidase of pig kidney (Smith and Spackman, 1951). In spite of the nomenclature, this enzyme also proved to have a very broad substrate specificity including peptides which carry various amino acids, other than leucine, at their amino end.

However, the cyclical structures of oxytocin and vasopressin possessing N-terminal half-cystine residues are resistant to its action. (Hill and Smith, 1957). Berankova et al, (1961) and Cihar et al (1961) compared the substrate specificity of oxytocinase and leucine aminopeptidase, and concluded that they are different enzyme systems. The same workers also reported that pregnancy serum oxytocinase is different and distinct from oxytocin-splitting enzymes in tissue extracts.

The results of the present study on the effects of inhibitors correlate well with the report by Tuppy and Wintersberger (1960) on a purified oxytocinase preparation. The outstanding feature is the profound inhibition by the metal-chelating agents 8-hydroxyquinoline and E.D.T.A. It is unlikely, however, that oxytocinase activity is dependent on dissociable metal ions, as the activity is not affected by dialysis. Furthermore, all metal ions tested in the present study and by Tuppy and Wintersberger (1960) either inhibited or did not affect the activity of oxytocinase. It is thus more probable that oxytocinase belongs to the group of metallo-enzymes with strongly bound metal ions.

Purification of pregnancy serum oxytocinase has not been achieved on a large commercial scale; the obvious difficulty is the limited supply of serum from women in late pregnancy.

VALIDITY OF THE CHEMICAL METHODS FOR THE ESTIMATION OF OXYTOCINASE

The synthetic substrate L-Cystine-di-β-naphthylamide (Fig. 21) introduced by Tuppy and Nesvadba (1957), shows a chemical similarity to the N-terminal amino acid sequence of oxytocin where the enzyme acts. It carries peptide bonds joining amino-free half-cystine residues with β-naphthylamine, which pregnancy serum oxytocinase seems to specialise in hydrolysing. On theoretical grounds, the enzymatic splitting of this substrate, which can be assessed by biochemical methods, should give a quantitative estimate of pregnancy serum oxytocinase activity.

Experimental proof for this view can be summarised as follows:

(a) In the present study it was found that the synthetic substrate is very slightly split by sera of non-pregnant women and of pregnant cow, pig, sheep, mice and rat, where oxytocinase has been reported absent or nearly so (Werle et al, 1950; Page, 1946). On the other hand, both oxytocin and L-Cystine-di-β-naphthylamide were readily split by sera of women in late pregnancy.

(b) As shown in the present study, the enzymatic splitting of L-Cystine-di-β-naphthylamide was affected in a similar, almost quantitative, pattern by the various inhibitors of oxytocinase. Tuppy and Wintersberger (1960) working with a purified oxytocinase preparation, reported similar findings.

(c) Fractionation of pregnancy sera by ammonium sulphate and paper electrophoresis in the present work showed that the recovery and distribution of the enzymatic activity towards L-Cystine-di-β-naphthylamine is similar to the oxytocinase activity as reported by Werle and Semm (1956). Identical distribution of the two activities was also reported by Page et al (1965), on starch gel electrophoresis, and by Hashimoto (1961) following column chromatography.

(d) Melander (1965) carried out parallel assays of the enzymatic splitting of oxytocin and L-Cystine-di-β-naphthylamide at the various stages of pregnancy. A linear correlation was found between the two sets of data in the last two trimesters of pregnancy (correlation factor "r" 0.94). However, this direct relationship was sometimes lost at low enzyme levels in non-pregnancy and early pregnancy sera, where the values of cystine aminopeptidase activity were higher than oxytocinase.

The second substrate introduced by Tuppy and Wintersberger in 1964 (S-Benzyl-cysteine-β-naphthylamide) was found in the present study to give parallel results, but was more readily split by pregnancy sera. Tuppy and Wintersberger (1964) reported that this new substrate was split 14.5 times faster than L-Cystine-di-β-naphthylamide. In our experience, this factor was only 10.0 on the average.

Berankova et al (1961) employed the synthetic peptides S-Benzyl-Cysteinyl Tyrosine for the biochemical estimation of pregnancy serum oxytocinase. The underlying principle

is the same, but the results could only be expressed semi-quantitatively by identifying the reaction products on a paper chromatogram.

LIMITATIONS OF THE CHEMICAL METHOD FOR ESTIMATION OF OXYTOCINASE

The use of L-Cystine-di-β-naphthylamide and other allied synthetic substrates for estimation of oxytocinase is based on a study of the exact mechanism of cleavage of oxytocin by the specific enzyme in human pregnancy sera, which acts as a specific "cystine" aminopeptidase. There is little doubt that oxytocin can also be inactivated by some other proteolytic enzymes (Croxatto et al, 1942; Bisset, 1962), which may not have a parallel effect on these synthetic aminopeptidase substrates. It is also quite possible that L-Cystine-di-β-naphthylamide may be cleaved by other aminopeptidase which are different from the specific pregnancy serum enzyme. This overlap of aminopeptidase substrate specificity (discussed by Nachlas et al, 1962) imposes a limitation on the use of the chemical method for evaluating "oxytocinase" activity in body fluids and tissue homogenates, other than pregnancy serum. Negative results in such experiments (e.g. in the case of amniotic fluid) indicate absence of the specific cystine aminopeptidase of pregnancy sera. Positive readings, on the other hand, unless backed by other evidence that the responsible enzyme is identical with the pregnancy serum

factor, cannot be interpreted as oxytocinase activity. Results on "tissue oxytocinase" estimations by this biochemical method (Melander, 1965) are thus very unreliable. In the present study, although figures for cystine aminopeptidase activity in placental extracts were quoted, we were only justified in reporting them because they were nearly identical with the results of the biological assay of "Placental oxytocinase". By both methods, the enzyme content in one gm. of placental tissue was approximately 4 times that of one ml. of late pregnancy sera. There is also some evidence from electrophoresis experiments (Page et al, 1961) that the responsible enzyme in placental extracts is identical with pregnancy serum oxytocinase.

As pointed out by Tuppy (1961) the low values obtained by the chemical method in non-pregnancy sera (0.3 mg. β-naphthylamine/100 ml. serum/hour in the present study) may be due to the non-specific splitting of L-Cystine-di-β-naphthylamide by an aminopeptidase present in all sera, and different from oxytocinase. The presence of such enzyme(s) is documented by the work of Behal et al (1963) and Green et al (1955), who reported substantial cleavage of the aminopeptidase substrate Leucine-β-naphthylamide following incubation with sera of men and non-pregnant females. Tuppy's views are also supported by Melander (1965) who found that the chemical method commonly gave relatively higher values than the biological method for oxytocinase in non-pregnancy and early pregnancy sera. Although most workers agree that oxytocinase is absent in

non-pregnancy sera, Page (1946) could detect some inactivation of oxytocin after prolonged incubation with non-pregnancy sera.

Another limitation for the chemical method is imposed by the method employed for the colour development of β-naphthylamine released by enzymatic activity. Sulphonamides and Sulphonurea compounds give the same colour reaction. The additional readings cannot be compensated for by the use of a zero time sample as a blank (Melander, 1965). The method is thus not suitable for assay of oxytocinase activity in the sera of pregnant women receiving those drugs.

Although the chemical method can be used to study the properties of the enzyme (e.g. pH activity range, effect of various inhibitors), it does not provide any data on the kinetics of the enzymatic inactivation of oxytocin by pregnancy sera such as reaction velocity, half-life time or Michaelis constant "Km". Under the conditions employed for estimation of oxytocinase by the biochemical methods, excess of the synthetic substrates is employed to ensure a zero order reaction, and not a first order curve. Moreover, the velocity constants are different with different substrates.

The Km value for oxytocinase was estimated by biological methods by Mendez-Bauer et al (1961) who gave a figure of 5 U/ml. (10^{-5}M/litre), and by Melander (1965) who reported a value of 1 U/ml. (0.2×10^{-5}M/litre). The

half-life value for oxytocin incubated with late pregnancy serum (or plasma) was given as less than one minute by Page (1946), 10 minutes by Dicker and Whyley (1959) and 15 minutes by Melander (1965). Under our conditions of incubation, 30 - 50% of oxytocin was inactivated in 10 minutes by sera of women in advanced pregnancy.

UNITS FOR OXYTOCINASE ASSAY BY THE CHEMICAL METHODS

Under the present conditions of incubation, the kinetics of the enzymatic splitting of the synthetic substrate L-Cystine-di-β-naphthylamide follow a zero order pattern. The amount of β-naphthylamine released per unit time could thus be employed to estimate the enzyme activity. For convenience, the unit actually employed in this study was calculated as mg. β-naphthylamine/100 ml. serum/hour. This is the original unit introduced by Tuppy and Nesvadba (1957).

Melander (1965) followed the recommendations of the International Union of Biochemistry in expressing oxytocinase activity by substrate hydrolysis rather than measurement of the reaction products. In zero order reactions one international unit of enzyme activity is defined as the amount which will catalyse 1 micromole of substrate per minute during the initial stages of the reaction. The units employed in this present study can be transformed into international units/ml. serum by multiplying by a factor of 0.58×10^{-3}. Klimek and

Pietrzycka (1961) and Fylling (1963) expressed their results in other units which have little to recommend them.

ADVANTAGES OF THE CHEMICAL METHOD OF OXYTOCINASE ESTIMATION

As compared to the biological method, the chemical method for oxytocinase estimation is less laborious and has higher accuracy and precision. With a good preparation two 4-point assays of oxytocin on isolated rat's uterus can be managed in one working day. By contrast, oxytocinase estimations by the colorimetric method can be done on three different specimens of sera simultaneously. Although colour development was usually performed on the next day, up to nine estimations could be managed in one working day. The fluorimetric method is even more simple and time saving.

The accuracy of the various methods can be deduced from the average standard error in recovery experiments. In the present study, the average error of oxytocin assay on isolated rat uterus in recovery experiments was 11.8%. In dialysis experiments, the colorimetric estimation of oxytocinase had a much smaller standard error of 1.0%. In duplicate estimations the difference between the two colorimetric readings was usually less than 2%, and rarely exceeded the 5% limit at low enzyme concentrations, which also indicates great precision of this method.

EVALUATION OF THE RESULTS OF OXYTOCINASE ESTIMATION IN NORMAL AND ABNORMAL PREGNANCY

The results obtained by the spectrophotometric method in the present study conform well with those of Muller-Hartburg et al (1959); Titus et al (1960) and Melander (1965). Table 29 compares the average figures for normal non-pregnancy and late pregnancy sera quoted in the various studies. The rise of enzyme levels by the end of pregnancy is thus in the order of 16 - 29 times above the non-pregnancy levels. As noted by Tuppy (1960) and confirmed by Melander (1965), the chemical methods probably give an unduly high estimate for oxytocinase activity in non-pregnancy sera; the true rise in oxytocinase activity may thus be higher than this.

In the present study it has also been established that the oxytocinase content is higher in sera obtained from patients with multiple pregnancy. Similar results were reported by Melander (1965) (on 6 patients), and by Glendening et al (1961) (number unspecified). In view of the prevailing idea that the enzyme arises from the placenta, this finding can be explained on the basis of increased placental mass in such cases. The low values recorded in subjects of moderate and severe pre-eclampsia could similarly be interpreted as a manifestation of placental insufficiency. The same finding was reported by Ichaliotis and Lambrinopoulos (1964), and is parallel to the deficient steroid hormone excretion in such patients (e.g. Roy et al,

TABLE 29

Author	Oxytocinase Level in Non-Pregnant Subjects	Oxytocinase Level in Advanced Pregnancy	Extent of Rise
Present Study	0.30	4.8	16 times
Muller-Hartburg et al, 1959	0.20	5.6	28 times
Titus et al, 1960	0.20	5.8	29 times
Melander, 1965	0.29	5.0	17 times

Serum (or Plasma) Oxytocinase Levels. All Figures are Calculated as mg. β-naph./100 ml. Serum/Hour

1963; Robertson and Maxwell, 1963). Page (1946) and Aragon (1948) employing biological methods for oxytocinase estimation, however, reported that the enzyme levels in tozaemic patients were not different from the normal. The low oxytocinase levels in toxaemic subjects could be advanced as an explanation of the increased oxytocin sensitivity of the uterus (Poseiro and Caldeyro-Barcia, 1958) and the exaggerated antidiuretic response (Dieckmann and Michel, 1935) to vasopressin in these patients. Riad (unpublished observations) however, employing Smyth oxytocin sensitivity test (1958), was unable to establish a significant difference in the uterine response between normal and toxaemic subjects in late pregnancy.

In cases of threatened abortion (16^{+} to 20 weeks of gestation) the enzyme levels were not significantly different from the levels in normal pregnancy at the same stage of gestation, even in patients who subsequently aborted within one week. Melander (1965) reported a similar finding. The same author also followed up cases of intrauterine death in the second half of pregnancy, and reported that the serum oxytocinase levels were still within the normal range 1 - 3 weeks after foetal death.

There are few reports in the literature about serum oxytocinase levels in cases of molar pregnancy. In addition to the case reported here, Hashimoto (1961) and Melander (1965) reported on two cases each. The serum enzyme levels in all cases fell within the normal range. It is thus

doubtful whether biochemical oxytocinase estimations will be helpful in diagnosing a following up case of molar pregnancy. The levels in the fluid expressed from the molar vesicles in the present study were found identical to the serum levels. This could be taken as further proof that the hydropic change in molar villi is due to the absorbtion by the active trophoblast in absence of foetal villous capillaries.

Glendening et al (1961) reported that serum oxytocinase levels (estimated biochemically) are not raised in cases of chorion epithelioma.

CHANGES IN SERUM OXYTOCINASE ACTIVITY IN NORMAL AND INDUCED LABOUR

In the present work, results of serial assays of oxytocinase activity in 14 patients revealed no significant difference between the late pregnancy levels and those obtained in early labour. A slight drop was observed in 4 patients, but did not exceed 15% in any of them. Itis thus difficult to attribute any role to the enzyme as a causative factor in initiating labour. The same conclusion was arrived at by Titus et al (1960).

The serum enzyme levels in cases of uterine inertia were also similar to those in normal labour. It is thus unlikely that abnormalities in uterine activity during labour are caused by altered enzyme levels. Babuna and Yenen (1966) however, reported that the enzyme levels are

higher than normal in cases of uterine inertia. This conclusion was not based on proper statistical analysis of their published data, which show that the enzyme level was less than the normal average in one out of 12 cases.

A rise in plasma (or serum) oxytocinase activity during infusion of oxytocin in pregnant women was reported by Mendez-Bauer (1961) and Tuppy (1961). In the present work, such a rise was established in 12 out of 39 patients. Neither the occurrence nor the extent of this rise was related to the dose of oxytocin administered, nor to the uterine response to oxytocin, as judged by the success or failure of induction. The only positive correlation was the frequent occurrence of this rise in patients who had short labours after a successful induction.

The rise in serum oxytocinase activity that was occasionally associated with oxytocin infusion could be explained in various ways.

(a) Oxytocin infusion induces enzyme formation. This is difficult to accept in view of the fact that the rise is in no way related to the dose administered.

(b) The oxytocin induced uterine contractions lead to release of the enzyme from some intrauterine source, e.g. the placenta or decidua. The frequent occurrence of this phenomenon in successful inductions with short labour, and presumably excessive uterine activity, lends support to this view.

The absence of correlation between the outcome of induction of labour by oxytocin and changes in the serum levels of oxytocinase confirms the report by Fylling (1963). In a later paper, the same author (1964) reported that a rise of oxytocinase was regularly noticed after a single intramuscular injection of oxytocin (Pitocin 10 I.U.) in early pregnancy (8 to 20 weeks). No similar experiments were performed in the present work. The usual indication for oxytocin infusion in early pregnancy is induction of cases of missed abortion, which are relatively uncommon. It must be mentioned that, as previously stated, estimates of oxytocinase activity in early pregnancy by the chemical method are not completely reliable.

CLINICAL VALUE OF OXYTOCINASE ESTIMATION

Pregnancy serum oxytocinase estimations by biological methods were employed by Page (1947) and Semm (1955) for the diagnosis of early pregnancy. In the present work, the values obtained by the chemical methods in early pregnancy showed a wide overlap with non-pregnancy levels. Single estimations were not reliable for the diagnosis of pregnancy before 16 to 20 weeks of gestation, by which time the diagnosis of pregnancy could usually be established by chemical methods. The available methods based on the detection of chorionic gonadotrophin hormone in urine by biological or immunological tests are thus far superior to oxytocinase estimation. It is possible,

however, that the latter assay may be of some use in excluding pregnancy in the follow up of patients suspected of having recurrence of vesicular mole or chorion epithelioma on the basis of high gonadotrophin excretion values.

The present study excludes the possibility of employing oxytocinase estimations as a method for assessing gestational age with any degree of precision. Individual variations around the mean value for any period of gestation are so wide (Table 21) as to make any such predictions of gestational age completely unreliable. These results are at variance with the work of Page (1946) and Semm (1955) who claimed that biological oxytocinase estimations were useful for this purpose.

The levels of oxytocinase in advanced pregnancy or the beginning of labour do not seem to reflect the pattern of uterine activity in labour and are similarly not useful in predicting the outcome of induction.

A potential use of serum oxytocinase estimation may be in differentiating maternal from foetal blood in late pregnancy. This is occasionally required in diagnosing the origin of bleeding in cases of antepartum haemorrhage of doubtful origin, and in cases of a "bloody" tap following attempted abdominal amniocentesis. The available chemical methods (based on identification of foetal haemoglobin) do not always provide a decisive answer. The levels of oxytocinase in foetal blood (0.4 mg. β-naphthylamine/100 ml. serum/hour) should be easily and decisively differentiated from maternal blood levels (4.8 units)

SITE OF ORIGIN OF PREGNANCY SERUM OXYTOCINASE

There is a certain amount of indirect evidence in this work as well as in reports by other workers, to indicate that the enzyme is formed by the placenta. This can be summarised as follows:

1 The enzyme is absent (or nearly so) in the sera obtained from men and non-pregnant females.

2 Serum oxytocinase occurs only in pregnant women and higher primates who have a haemochorial type of placentation. It is absent in lower animals where other forms of placentation do not allow such a close contact between the trophoblast and the maternal organism.

3 The distribution of the enzyme in the products of gestation is also suggestive of a placental origin. The enzyme is nearly absent in the amniotic fluid and in the foetal circulation, in contrast with the high and rising levels in maternal serum throughout pregnancy. Placental extracts are a rich source of oxytocin-splitting enzymes.

4 The rising levels of oxytocinase in maternal sera throughout pregnancy show an analogous pattern to the curve of placental growth, and are not unlike the progress curves of other enzymes (e.g. histaminase) and steroid hormones reported to arise in the placenta. The high serum levels in cases of multiple pregnancy are also suggestive of a correlation with placental size.

5 Werle (1960) employing biological methods for oxytocinase assay, presented evidence that the oxytocin-

splitting enzymes in a variety of mammalian tissues, are different from the specific oxytocinase in human pregnancy sera. Tissue oxytocinases proved to be less stable and had different pH optima and inhibitor spectra. Similar observations were made by Rychlik (1964) employing biochemical methods.

6 Semm (1963) incubated slices of placental tissue with the synthetic substrate L-Cystine-di-β-naphthylamide under favourable conditions for oxytocinase activity, and localised the β-naphthylamine release by a histochemical technique. He reported that the enzyme is localised in the syncitial layer of the trophoblast. The limitations of the use of the chemical methods for "oxytocinase" estimation in tissues have been discussed on page 142.

7 Glendening et al (1961) reported that placental extracts subjected to vertical starch gel electrophoresis showed bands of cystine aminopeptidase activity similar to those obtained with late pregnancy sera. No similar bands could be obtained by electrophoresis of extracts of the myometrium, ovary, decidua or Fallopian tubes, in spite of their substantial cystine aminopeptidase activity.

In the present study, attempts at direct proof of the placental origin of the enzyme have not been successful.

1 The effect of inhibitors on pregnancy plasma oxytocinase (measured biologically) is somewhat different from the results on oxytocin-splitting enzyme(s) in placental extracts. Under similar conditions of incubation, the placental factor is not inhibited by metal-chelating agents

in contrast to the plasma enzyme.

2 The enzyme content of the uterine vein blood (which is the main route for drainage of the chorio-decidual space) is not higher than the levels in systemic (arm vein) blood obtained simultaneously. Such a gradient has been reported with oestrogens (Roy, 1962), progesterone (Zander and Von Munstermann, 1954) and histaminase (Swanberg, 1950), all of which are thought to arise in the placenta.

3 The slow decline in serum oxytocinase activity after expulsion of the placenta at term delivery is difficult to reconcile with a placental origin, and is different from the rapid clearance of oestrone and oestradial progesterone and chorionic gonadotrophin following delivery. Melander (1965) reported the same finding, and proposed that the slow decline of the serum enzyme levels after delivery could be due to the continued production by decidual remnants. In the present study, the serum oxytocinase levels in subjects receiving an oral gestagen (which produces changes in the endometrium not unlike the decidua of pregnancy) were not higher than the levels in the normal controls.

THE PHYSIOLOGICAL ROLE OF PREGNANCY SERUM OXYTOCINASE

It has been suggested that pregnancy serum oxytocinase is a protective mechanism which helps to regulate the levels of oxytocin in blood, and to protect the human pregnant uterus against sudden outbursts of neurohypophysial activity

(Semm, 1961; Fylling, 1964). There is very little evidence to support this hypothesis. The high levels of the enzyme in late pregnancy certainly do not suppress the uterine response to injected oxytocin. On the contrary, the sensitivity of the human uterus to oxytocin rises throughout pregnancy (Pose and Caldeyro-Barcia, 1958) despite the rising levels of oxytocinase. Melander (1965) also found no correlation between the serum enzyme levels and the onset of premature labour. In the present study, the uterine response to oxytocin infusion in late pregnancy (as assessed by the doses of oxytocin required for induction of labour, and by the outcome of such induction) was in no way related to the serum enzyme levels.

Similarly, Massi et al (1965) reported that in late pregnancy the uterine effects of infusion of Desamino-oxytocin (which is not inactivated by serum oxytocinase) do not last longer than oxytocin.

Furthermore, even in human males and in lower animals who lack the serum oxytocinase enzyme, there seem to be very efficient mechanisms for eliminating oxytocin from the circulation. As seen in Table 3 (page 32), the half-life of oxytocin in the rat, rabbit, sheep and human males, is not substantially different from the values obtained in late human pregnancy, and does not exceed a few minutes. It must be recalled, however, that the half-life values of pituitary peptides in the circulation do not reflect on the duration of the uterine response to oxytocin or the renal effects of vasopressin, which last much longer (Sica-Blanco

and Sala, 1961; de Wardener, 1957).

These discrepancies may be due to:

1 Half-life experimental data were based on the administration of large doses of oxytocin and vasopressin. If we accept that the hormones are largely eliminated in the body by enzymatic inactivation, these figures would not necessarily apply to the low physiological levels at which enzymatic cleavage proceeds more slowly.

2 The initial rapid clearance of the hormones from the circulation may represent redistribution in the various fluid compartments in the body rather than final inactivation. Fitzpatrick (1961) reported that during one circulation of the blood in the human male and the female sheep, a considerable proportion of intravenously injected oxytocin seemed to diffuse beyond the vascular compartment. Similar observations were recorded in the rat (Ginsburg and Smith, 1959). Aroskar et al (1964) also found that following intravenous injection of tritium-labelled oxytocin in the rat, radioactivity was widely distributed in the body, even in areas of low vascularity, e.g. brain substance and muscle.

3 The sustained uterine and renal effects of posterior pituitary hormones may simply represent rates of recovery of the target organs to the resting state after the hormones have been eliminated by excretion

or enzymatic degradation.

In view of the finding that the pregnancy serum inactivates vasopressin as well as oxytocin, it might be speculated that the enzyme system is more concerned with water and electrolyte changes in pregnancy than with regulating uterine contractility. It is possible that the high circulating levels of water and electrolyte retaining steroids (aldosterone as well as placental steroids) necessitates adjustment of the antirenal effects of vasopressin. This view can be tested by comparing the renal effects of vasopressin (injected or produced in the test subjects by dehydration) in pregnant women as compared to a group of non-pregnant controls. In a recent work, Torres et al (1966) reported differences in the electrolyte excretion response to injected 8-arginine vasopressin in pregnant women, as compared with non-pregnant subjects. Intravenous infusion of arginine vasopressin in mid-pregnancy led to a marked increase in urinary sodium excretion. In the last trimester this effect could not be reproduced, possibly as a result of the high levels of circulating "vasopressinase".

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